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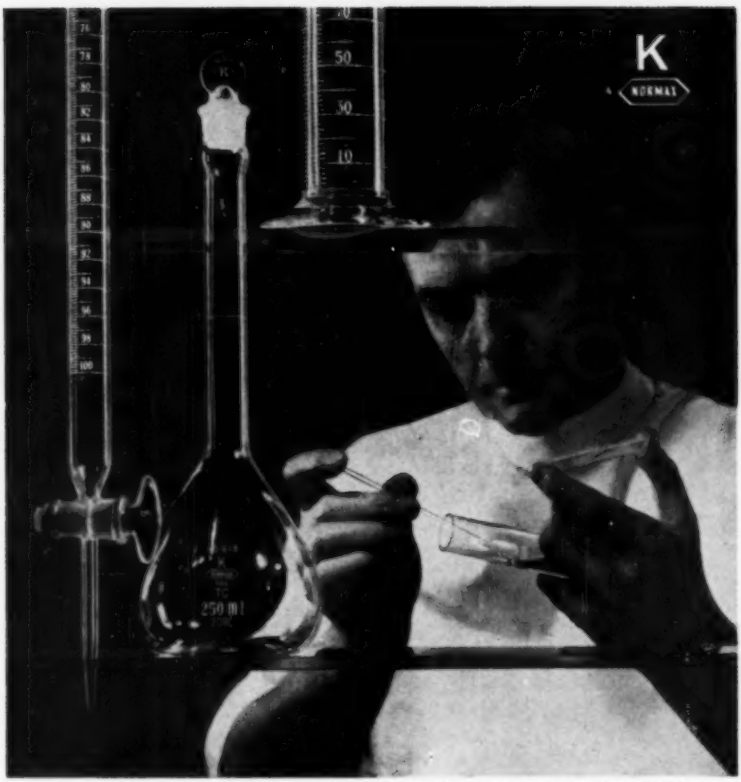

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XL | *DECEMBER, 1951* | NUMBER 12 CONSECUTIVE NO. 24

Fate of Iodine in the Body*,†,‡

By C. P. LEBLOND§

IODINE took on biological significance 100 years ago when this element was used successfully to treat a condition now known as exophthalmic goiter. Recent research has revealed that iodine is more than a drug. As a component of the molecule of the thyroid hormone, it plays a fundamental role in physiology, the nature and intricacies of which are now being investigated by use of its radioactive isotopes.

In the present paper, the behavior of iodine in the animal organism will be described. The body iodine comes from the food, and available evidence indicates that most of the iodine present in foods of vegetable origin is in the form of its ion, iodide, while food of animal origin may contain some organically bound iodine as well as iodide. It is well known that iodide administered by mouth is readily absorbed since it reaches the thyroid gland within a few minutes after ingestion. In fact, at the end of a given interval after oral ingestion, about the same amount enters the thyroid gland as after subcutaneous administration (1). Less, however, is known regarding the absorption of organic iodine. From work done on diiodotyrosine and thyroxin it can be predicted that some of the organically bound iodine may be absorbed as such and the rest broken down to iodide, thus augmenting the amount of inorganic ion available for absorption. Therefore, it is assumed that most of the iodine normally entering the body is, or will be, in the latter, i. e., ionic form. Accordingly, investigations here reported are limited to the fate of this ion in the animal organism. The evidence will be taken up in the following subdivisions: (*a*) methods of investigation; (*b*) distribution of extra-thyroidal iodide; (*c*) metabolism of iodide within the thyroid gland.

METHODS OF INVESTIGATION

Three main methods were used. Since the work was carried out in rats, the minute amounts of iodine physiologically present in the organs and tissues of this small laboratory animal demanded a very sensitive method of *iodine estimation*. Chaney's method (2) was therefore adapted to the needs of this Laboratory by J. Gross and found to be sufficiently sensitive, since amounts as small as one-hundredth of a microgram could be accurately determined. Purified reagents and carefully controlled conditions proved essential. The technique consists of sulfochromic oxidation (I^- to IO_4^-), followed by reduction with phosphorous acid (IO_4^- to I_2) and imme-

* Received October 9, 1951, from the Department of Anatomy, McGill University, Montreal, Quebec, Canada.

† Presented to the Scientific Section of the A. Ph. A., Buffalo meeting, August, 1951, by Dr. C. P. Leblond, recipient of the third (1950) Chilean Iodine Educational Bureau Award for outstanding research in the chemistry and pharmacology of iodine.

‡ The work reported in this paper was supported by a grant from the National Research Council of Canada.

§ The work of the author was done with several collaborators, particularly, P. Sue, W. Mann, and J. Gross.

We are indebted to the National Research Council of Canada for continuous support throughout these investigations. Thanks are due to Miss J. Cambron for technical assistance.

diate distillation of the resulting iodine which is collected as iodide in a sodium arsenite solution. Subsequently, the iodide is measured by its power to catalyze the transformation of the ceric into the cerous ion by the arsenite. Since the yellow color of the ceric ion decreases as this ion is reduced, the consequent rate of change of light absorption may thus be used to calculate the amount of iodide present.

The second method used was that of *paper chromatography*. Iodine-containing substances can be identified with great accuracy by their location on two-dimensional paper chromatograms using formic acid acidified butanol in the first dimension and ammoniacal butanol in the second dimension (3). This method was used to identify the iodinated substances present in the thyroid gland, as well as in other organs and in excreta.

The third and most fruitful tool used in this investigation was *radioiodine*. The radioactive isotope of iodine, I^{131}, was employed to reveal the fate of natural iodine, I^{127}, in the body. I^{131} and I^{127} exhibit identical chemical behavior since both have 53 electrons around their atomic nuclei and both are consequently called "iodine." The difference in nuclear weight (127 *versus* 131) is so slight as to be detectable only as a minute difference in a limited number of physical properties. The preparation of uranium 235 for the atom bomb required the separation of two such isotopes. The tremendous difficulties encountered in that undertaking have been sufficiently publicized so that the present point will be readily understood. The lack of chemical difference between I^{131} and I^{127} is most accurately demonstrated by two modern techniques, namely, isotope dilution and paper chromatography:

1. The first of these reveals that compounds labeled with radioiodine can be repeatedly crystallized or precipitated without change in the amount of radioactivity per mg. of compound, i. e., without altering the specific activity of the compound under investigation (4, 5).

2. By paper chromatography it can be shown that radioactive thyroxin obtained by chemical or biological synthesis reaches, on the paper, a location identical to that of commercially available thyroxin (3, 6). This is also true of other substances labeled with I^{131}.

However, the identity of the chemical behavior of I^{127} and I^{131} does not necessarily imply an identity of physiological behavior. Two more conditions must also be met before experiments with radioiodine can be used to indicate what the true behavior of iodine is under any given set of conditions, namely, the radio-chemical effect must be negligible and the weight of iodine administered must be small. (*a*) The work of Skanse on the problem of the radio-chemical effect reveals that in the chick, a dose of 10 μc. or more may have damaging effects (7). Smaller doses should, therefore, be used for physiological work. Whether or not a dose is safe depends on the sensitivity of the tissue to the radiation, as well as on the local concentration of radioactivity. The concentration is often spotty and a localized radio-chemical effect would usually be hard to detect. Thus, theoretical considerations are of little value in predicting whether or not a radio-chemical effect will be present. Therefore, after results have been obtained with a given amount of radioactivity, it is advisable to repeat the experiment several times using smaller and smaller doses until the lowest possible radioactive dose showing the results is reached. This procedure reduces but does not necessarily rule out the possibility of interferences due to radio-chemical damage. (*b*) The amount or weight of iodine injected must be sufficiently small to produce no significant increase in the amount of circulating iodide if conditions similar to the physiological are to be obtained. This requirement is readily met since radioiodine is prepared by transmutation of tellurium under neutron bombardment. Theoretically, the number of resulting iodine atoms will be relatively small, even though the sample may be intensely radioactive. In fact, the iodine content of the material available, which is of the order of 1 μg./mc., is mainly due to a slight contamination from the natural iodine present in the reagents used in the extraction process.

Several other points must be kept in mind when working with this isotope. The radioiodine used in scientific laboratories is prepared in the form of the iodide ion by the three main suppliers (Atomic Energy Commissions of the U. S. A., Canada, and England). However, solutions that are several weeks old may also contain iodine (I_2) as a result of the action of the radiations on the solution itself. (This may be shown by paper chromatography.) When such material is administered to animals, reactions between I_2 and the proteins at the injection site may occur, thus giving rise to artifacts. In practice, it is safe to use only fresh solutions of I^{131} or to add an agent to old solutions to reduce I_2 back to iodide.

A second major danger in using radioactive iodide is the possibility that the iodide entering the body in radioactive form may be exchanged with the nonradioactive iodine present in organic molecules in organs and tissues. Such exchange reactions are unlikely to occur in the body since it is known that at *p*H 7, in the absence of active oxidants, the iodide ion does not exchange with the iodine atoms of thyroxin and diiodotyrosine (8–10). On the other hand, exchange may occur, especially with diiodotyrosine, under conditions of acidity, high temperature, or in the presence of oxidative reagents (9). This possibility complicates the processing of tissues in which an attempt is being made to identify iodine-containing compounds, since acid media, heat, and conditions favoring oxidation must then be avoided. (There are, however, types of experiments in which the occurrence of exchanges would not influence the interpretation of the data and, therefore, acid media and heat may be used.)

The techniques used for work with radioiodine are well known, namely, the Geiger counter to carry out quantitative estimations, and the "radioautographic technique" to reveal the location of radioactive substances in histological sections (11). Good localization may now be obtained, particularly with the "coating method" by which a histological section is coated with photographic emulsion in the dark. After exposure for various lengths of time, this preparation is developed as in ordinary photographic work. In the resulting "radioautograph" black silver granules overlie those sites which contain the radioactive material retained in the histological sections.

DISTRIBUTION OF IODIDE OUTSIDE THE THYROID GLAND

Wallace and Brodie (12) showed that iodide distributes itself throughout the extracellular spaces, and, in so doing, behaves like other halogens and the halogen-like thiocyanate ion. This fact was generally confirmed with radioiodide (1) with two important exceptions, that is, the excretory organs, and of course, the thyroid. In the excretory organs, iodide progresses beyond the extracellular spaces and enters the cells. Thus, the presence of more radioiodide in the liver than could be accounted for by the known capacity of the extracellular space of this organ may be taken to indicate the entry of this ion into the cell prior to eventual passage into the bile. Similar results were obtained with kidney and stomach (1). The fact that large quantities of iodide are excreted by the kidney after iodide administration (13) has been amply confirmed by clinical studies. On the whole, the quantity of iodide excreted in man reflects the amount which is not taken up by the thyroid gland (14–17, Table I).

TABLE I.—PERCENTAGE OF ADMINISTERED DOSE OF LABELED IODIDE EXCRETED IN URINE AFTER ORAL INGESTION[a]

	Skanse (17)		Oshry and Schmidt (18)	
	No. of Cases	Range of Radioiodine Excretion, 48 Hr.	No. of Cases	Range of Radioiodine Excretion, 24 Hr.
Normal	15	52.7–84.1	23	42–80
Myxedema	6	72.4–91.7	10	48–93
Hyperthyroid	26	6.2–32.3	34	5–43

[a] After Raben and Astwood (16).

This problem was systematically examined in rats by comparing animals fed Purina Fox Chow, a commercial diet supplying a more than adequate iodide intake, with others fed a diet containing little iodine. The elimination of radioiodine into the urine within twenty-four hours after administration was of the order of 90% in the Purina-fed rats and 20% in the iodine deficient rats, while corresponding thyroid uptakes were, respectively, 8% and 55%. Thus, the urinary loss of iodine was low when the supply of this element was low, and, as in man, there was an inverse ratio between urinary excretion and thyroid uptake. [In all cases, the iodine of the urine was in the form of iodide (19).]

The excretion of iodide into the stomach was first reported in 1929 (20) and more recently confirmed with radioiodide (1, 8, 15, 21–24). Since the iodide ion is concentrated in the stomach like the bromide and chloride ions, the halogen nature of these substances is probably responsible for their gastric secretion. Furthermore, the gastric iodine is not recoverable in the feces and, therefore, must be reabsorbed farther down the gastrointestinal tract. Thus, the excretion of iodide into the stomach may be considered as a mechanism for maintaining the iodide store by shunting it through the portal before returning it to the systemic circulation, where of course the kidney and also the thyroid can act on it. Thus the gastric passage delays a too rapid loss of iodine into the urine.

While much of the iodine entering the body goes rapidly into gastric juice and urine, the concentration of radioiodine in these locations gradually decreases with time. In contrast, the skin shows a concentration of radioiodine which continues to increase with time. Thus, at seventy-two hours after injection of radioiodide, the skin contains 38% of the radioactivity present in the body (25).

Recently this finding was reinvestigated along a different line. Iodine estimations were carried out in the organs of rats fed Purina Fox Chow. Under these conditions, the concentration of iodine was low in all organs investigated, except thyroid gland, stomach and skin. The thyroid gland contained 100–200 mg. of iodine per 100 Gm. of tissue, or roughly 30,000 times more than most of the other organs, while the stomach and skin, and also the urine, contained about 10 times more than the other organs (Table II). In order to determine in what skin component the iodine was present, an area of skin was shaved and the iodine content of both hair and remaining skin was determined. The high concentration of iodine was thus traced to the hair, there being about 100 times as much iodine found there as in most of the other organs (26).

TABLE II.—MEAN IODINE CONTENT OF ORGANS AND TISSUES IN ALBINO RATS FED AN ADEQUATE IODINE INTAKE DIET

	Iodine Concentration (Mg./100 Gm.)
Thyroid gland	129.800
Plasma	0.006
Liver	0.006
Kidney	0.004
Muscle	0.002
Thymus	0.005
Ovary	0.004
Uterus	0.005
Stomach	0.053
Skin	0.045
Urine	0.039
Hair	0.437

The next step in this investigation was to identify the chemical form of the hair iodine. Skin areas of normal and thyroidectomized rats were shaved, and, after a time interval sufficient to allow newly growing hair to appear, radioiodine was injected. Three days later, the regrown hair was plucked, weighed, and repeatedly extracted with water-saturated butanol at a neutral *p*H. The pooled butanol extracts were found to contain radioactivity. By paper chromatography, this radioactivity proved to consist exclusively of iodide, whether normal or thyroidectomized animals were used. These results were taken to signify that iodide is taken up by the growing hair from the circulation and deposited there as such (26). It may be concluded that the iodide ion is readily excreted by the kidney, liver, and especially the stomach, which seems to have a rather specific affinity for this halogen. Iodide is also taken up by growing hair. Of course the most striking uptake occurs in the thyroid gland.

THE METABOLISM OF IODIDE IN THE THYROID GLAND

In general, recent findings of tracer work are interpreted on the assumption of a "steady state." An organ is said to be in a steady state with respect

to one particular substance or metabolite when the amount of this substance or metabolite remains constant, and its rate of appearance is equal to its rate of disappearance. Many phenomena have been interpreted on this assumption, but its truth has generally not been proved. In fact, there probably are few phenomena which do not show a change in rate with the alternations of rest and wakefulness, or food intake and digestion.

The thyroid gland extracts iodine from the blood as a preliminary step in the synthesis of its iodinated hormone. To determine whether or not this gland was in a steady state with respect to iodine, its uptake of radioiodine was examined in rats at six different times throughout a twenty-four-hour period. The results revealed that the thyroid handles iodine with clocklike regularity. In fact, all the activities of the thyroid gland, which are now to be described, occur simultaneously, continuously, and at constant rates (27). For the sake of clarity these activities will be described under five headings:

(*a*) The entry of iodide from the circulation.

(*b*) The synthesis of thyroglobulin from iodide in the apex of the thyroid cells.

(*c*) The subsequent deposition of thyroglobulin in the colloid of the thyroid follicles.

(*d*) The hydrolysis of thyroglobulin yielding thyroxin and a number of other iodinated substances.

(*e*) The passage of thyroxin into the circulation.

The Entry of Iodide from the Circulation.—Many years ago, it was claimed that iodide was present in the thyroid gland (28). The implication was that this iodide was the form in which the circulating iodine entered the gland. These results were challenged, and various contributions to the debate (29–32) had not completely clarified the situation when the first studies with radioiodine showed the entry of this element into the thyroid gland (33–35). While the entry of iodine had already been seen without the help of radioactivity by Marine (36), the recent work showed that the newly entering radioiodine was stored in the form of the iodide ion (1, 3, 7, Fig. 1); but, as time elapses, it is transformed into organic iodine (references 37 and 38, dog experiments). However, the doses of iodide used in these pioneer experiments were far greater than those now considered physiological. In the rabbit, Lein (39), using a nearly physiological dose of 35 μg. of labeled iodine, also saw the radioactivity entering the gland as iodide and then undergoing organic binding. When, however, truly physiological doses of radioiodine were used, the sequence of events was obscured by the rapidity with which the radioactivity was bound into organic material. Thus, even at thirty minutes after injection of I^{131}, the specific activity of the iodide in the gland was less than that of the organic constituents (40). Astwood and collaborators (41–43), using physiological amounts, were able to approach the problem in an indirect manner. They made use of McGinty's finding of the entry of radioiodine into the thyroid after treatment with thiouracil or similar antithyroid drugs (44), and found that under these conditions the material in the gland consisted of radioiodide. It was emphasized that the thyroid can maintain an iodide concentration higher than that of the circulating blood, the ratio of thyroid to blood iodine averaging 25 in the normal animal and 280 in animals with hyperplastic glands (43). Somewhat higher values were obtained elsewhere (45, 46). It may be concluded that iodide enters the thyroid gland and is at first stored there as such. The entry and storage of iodide have since been confirmed by paper chromatography, as may be seen in Figs. 6–11 (3).

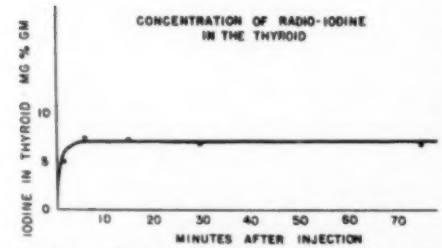


Fig. 1.—Curve of the concentration of radioiodide in the thyroids of rats sacrificed in groups of six at various time intervals after intravenous injection of 0.75 mg. of labeled iodide per rat.

When such a large dose of iodide is administered, almost all the material entering the gland is present as iodide.

The ability of the gland to collect iodide is limited, since a plateau is soon reached as shown in this figure. (Leblond and Sue, reference 8.)

Two methods may be used to estimate variations in the ability of the gland to concentrate the iodide ion, namely: (*a*) Determinations of the amount of radioiodide entering the thyroid gland of animals treated with the antithyroid drugs, such as propyl thiouracil (42, 43); (*b*) estimation of the I^{131} in the thyroid after injecting a large dose of radioiodide intravenously and quickly sacrificing the animals so as to anticipate any significant organic binding of iodide. The routine used here consists of the intravenous administration of 1 mg. of labeled iodide into mature albino rats, which are then sacrificed one-half hour later—a method used in earlier experiments on the subject (8). This method may be utilized to investigate whether or not the uptake of iodide by the thyroid is *under hypophyseal control*. Indeed, lower radioiodide uptakes were thus observed in hypophysectomized animals and higher ones in thyrotrophin-treated than in normal controls (8). That the iodide-concentrating ability of the thyroid gland is under hypophyseal control was also the conclusion recently reached by Greer and VanderLaan using animals given antithyroid drugs (quoted in reference 16).

Recently, the effect of stress was examined by means of injections of large doses of radioiodide followed by sacrifice of the animals one-half hour later. It was found that the iodide-collecting ability of the thyroid gland was reduced under the influence of stress, presumably through a decrease of thyrotrophin output by the hypophysis (47).

In conclusion, the thyroid gland possesses a very efficient mechanism for extracting iodide from the blood. Iodide enters the gland as such. Usually this iodide is rapidly transformed into complex substances. When an excess of iodide is available, a large amount may remain in the gland for some time before organic transformation occurs.

The Synthesis of Thyroglobulin in the Apex of the Thyroid Cell.—It has long been known that treatment of proteins with elemental iodine, I_2, results in the formation of iodo-proteins. Oswald showed that iodine substitution in the tyrosyl groups yielded diiodotyrosyl radicals without affecting the original peptide linkages (48). The findings by Harington and Barger (49) of diiodotyrosine in thyroid hydrolysates led these authors to postulate that the formation of the thyroidal iodo-protein, thyroglobulin, arose from an iodination similar to that observed by Oswald *in vitro* (50). This hypothesis received strong support from the investigations of von Mutzenbecher, who showed that iodination of casein with I_2 led to the formation not only of diiodotyrosine but of thyroxin as well (51). Thus, from iodinated casein (and other iodo-proteins as well, reference 52) the two main iodinated components of thyroglobulin could be isolated. Since protein iodination takes place at the physiological *p*H of 7.5, it is now generally believed that such a process accounts for the formation of thyroglobulin.

Iodide, to react with a protein, must be oxidized to IO^- or rather to I_2 (53). Recently, the presence of I_2 in thyroid tissue was suggested by its appearance in paper chromatograms of thyroid extract (3). This proof should not be considered conclusive, due to the difficulty of obtaining adequate controls in this type of experiment. In any case, the formation of I_2 or other suitably oxidized form of iodine would require an enzyme system including some type of peroxidase. Keston (54) reproduced such a system *in vitro* by adding radioiodide to unpasteurized milk. Milk is known to contain a protein, caseinogen, as well as xanthine oxidase and peroxidase. Nothing happened in the presence of radioiodide unless xanthine was added as a substrate, after which 67% of the radioactivity was bound into an organic form. Thiourea, which inhibits iodination in the thyroid of living animals, also prevented this phenomenon. Presumably thiourea combines with I_2 at a faster rate than proteins do, and therefore no iodo-protein appears.

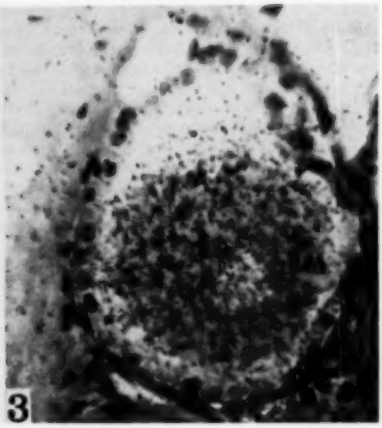
The presence of peroxidase activity in thyroid tissue has been claimed by Dempsey on the basis of histochemical findings (55), and by de Robertis and Grasso who used a supravital technique to reveal that thyroid tissue could transform iodide into iodine *in vitro* (56). Glock denied the presence of a peroxidase in tissue extracts (57). Possibly Glock's failure may be ascribed to his use of thyroid homogenates. It is known that iodine may be transformed into thyroglobulin in the presence of thyroid slices but not of thyroid homogenates (58). Homogenization may have resulted in an inhibition of the thyroid peroxidase. If this were the case, Glock's negative results would be readily understandable. At any rate, it is necessary to postulate the presence in the thyroid cell of an oxidative mechanism for the conversion of iodide to a form capable of reacting with tyrosyl groups. A peroxidase enzyme system would be suitable for this purpose.

Two of the iodinated amino acids bound in peptide linkage in thyroglobulin, namely, diiodotyrosine and thyroxin, have been mentioned above. Recently, hydrolysates of thyroglobulin were found to contain monoiodotyrosine (59), as well as a substance referred to elsewhere as compound 1 (3), and several unidentified substances (60, 61). Compound 1 has now been isolated (60). These substances are all bound into thyroglobulin, thyroxin itself being present as thyroxyl residues. It has been suggested that thyroxyl radicals arise from the oxidative coupling of two diiodotyrosyl radicals with the elimination of ammonia and pyruvic acid—a phenomenon which could take place under conditions of oxidation such as are afforded by the presence of I_2 (49, 50). Some support for this hypothesis was offered by von Mutzenbecher, who observed the conversion of diiodotyrosine itself into thyroxin *in vitro* (51). Studies of the respective specific activities of diiodotyrosine and thyroxin in thyroid hydrolysates at various time intervals after injection of radioiodide supported the theory that diiodotyrosyl radicals were the precursors of the thyroxyl radicals (62, 63). However, the separation of the two substances was unsatisfactory in these experiments, since it was later shown by paper chromatography that the so-called diiodotyrosine and thyroxin fractions each contain several iodinated substances.

While much remains to be learned about the mechanism of thyroglobulin formation, recent studies with the radioautographic technique revealed the site of this formation.

The thyroid gland is made up of a large number of tiny glandular units or follicles, of which there are up to 100,000 in the rat (64). Each follicle consists of an outer sphere made up of a continuous layer of epithelial cells enclosing a viscous fluid, the colloid. Separation of cells and colloid by centrifugation has shown that most of the gland iodine is in the colloid (65, 66). This iodine is in thyroglobulin, since the colloid of histological sections intensely absorbs ultraviolet rays at 2,800 Å., a band corresponding to thyroxyl and diiodotyrosil radicals, while the cytoplasm of the cells absorbs weakly in that band (67). Thus, there would be much thyroglobulin in the colloid and little, if any, in the cells. Another approach to this problem was based on histological observations showing that the colloid stains by means of the periodic acid-fuchsin sulfurous acid technique (68). This technique seems to stain thyroglobulin itself (whose carbohydrate moieties may react with periodic acid), as shown by the following experiment. Smears of purified thyroglobulin with a thickness of 5 μ, i. e., one equal to that of histological sections, were stained by this technique. The intensity of the stain was comparable to that observed in the colloid present in thyroid sections. It was concluded that not only was thyroglobulin present in the colloid, but very little else must have been there, and, therefore, the colloid must essentially consist of thyroglobulin (69).

The appearance of new thyroglobulin may be studied with radioiodine. When rats fed an adequate iodine intake are sacrificed one hour after injection of radioiodine, the radioautographs show an accumulation of photographic granules over the thyroid cells (Fig. 2). On close examination, this reaction could be assigned to the inner portion of the cell, that is, the so-called "apical region," which is the part of the cell closest to the colloid (70). Therefore, the iodide entering the thyroid cell from the outside must have first traveled to the cell apex. There the iodide is incorporated into a substance



Coated radioautographs of sections of thyroid from rats injected with 20 μc. of radioiodide and sacrificed either at one or at twenty-four hours after injection. Sections were stained with hematoxylin and eosin. The location of the radioactivity is indicated by the presence of black granules. The radioiodine thus detected may be shown to consist of newly formed thyroglobulin. (Leblond and Gross, reference 70.)

Fig. 2.—One hour after radioiodide injection. The radioactivity is in the apical portions of the cells. Note the extent of fog in the upper left corner.

Fig. 3.—Twenty-four hours after radioiodide injection. The radioactivity is now located throughout the colloid.

insoluble in the reagents used for histological processing. This substance cannot be iodide or any of the usual small iodine-containing organic molecules, since all these substances are readily extractable with either the aqueous (fixative, stain solutions, etc.) or the fatty (dioxane, alcohol, xylol) solvents used in making the sections. The only remaining iodinated substance known to exist in the thyroid gland is thyroglobulin, which is indeed rendered insoluble by histological fixation. Furthermore, soon after administration of radioiodine the radioactive material of the thyroid gland is precipitable with half-saturated ammonium sulfate, as is thyroglobulin. It may, therefore, be concluded that the autographic reaction observed in thyroid follicles indicated the presence of newly formed thyroglobulin (70).

Thus thyroglobulin must form from iodide in the apical region of the thyroid cells. It is well known that this area frequently shows the presence of minute cytoplasmic droplets containing a material which stains like the colloid. Recently, it was possible to show that the apical droplets, when present, stain with the periodic acid-fuchsin sulfurous acid technique and therefore were presumed to consist of thyroglobulin, like the colloid itself. Since these droplets originate in the Golgi region of the cells, presumably from antecedents which are at, or beyond, the limit of microscopic visibility (71), it is likely that the newly formed thyroglobulin made visible by radioiodine in about the same area also originates in the Golgi region (70).

The Deposition of Thyroglobulin in the Colloid.—The time spent by new thyroglobulin in the apical region of the cell must be rather short. In fact, when thyroid glands are activated by thyrotrophic hormone, the length of stay in the cells may be so short that at no time after injection is there enough new thyroglobulin in the cells to produce a clear-cut radioautographic reaction there.

At any rate, when rats fed an adequate iodine intake are sacrificed twenty-four hours after injection, the cells no longer show an autographic reaction, whereas the colloid does [(70), Fig. 3]. Practically all of the follicles exhibit a colloid reaction at that time. In other words, the thyroglobulin which was mainly present in the cells soon after injection (Fig. 2) has now passed into the colloid.

It is interesting to know whether the thyroid cell secretes thyroglobulin only in the direction of the colloid or also releases some directly into the circulation. This problem was approached by sacrificing rats with very active thyroids within fifteen minutes after intravenous injection of radioiodide. Under these conditions radioautographs showed the radioactivity to be in the colloid, while estimations with the Geiger counter revealed that up to 10% and more of the administered dose of radioiodide was present there. On the other hand, no radioactivity was organically bound in the blood. Thus, the secretory activity of the cells was exclusively oriented toward the colloid (70).

Examination of individual follicles after radioiodide treatment revealed the presence of radioactivity in all of them. This was true whether the radioactivity was in the cells or, at twenty-four hours, in the colloid. The demonstration of the existence of a steady state (27) makes it possible to conclude that at all times all follicles are active in building up thyroglobulin and depositing it in the colloid. This conclusion may be broadened even

further, since the follicles showing cellular localization of new thyroglobulin reveal that, at any given time, every single cell is active. Presumably, therefore, all thyroid cells are continuously elaborating thyroglobulin.

While all follicles show some synthesis of thyroglobulin, the more central ones usually make and deposit thyroglobulin more rapidly than do those situated just under the gland capsule or in the isthmus. It appears that each follicle maintains its own rate of formation of thyroglobulin. However, in a given follicle all cells tend to have an identical rate of activity.

Whatever the rate of entry of organic iodine into the colloid of the follicle lumen is, maintenance of the steady state makes it imperative that it be equal to the iodine output. It is, therefore, necessary to examine how the material may leave the gland and enter the circulation.

The Enzymatic Hydrolysis of Thyroglobulin.—In ascidians, the material secreted into the large single lumen of their primitive thyroid gland (thyroglobulin?) migrates through the passage connecting this lumen with the gastrointestinal tract. Presumably, the material is then digested in the intestine and the fragments absorbed. Thus, the circulating thyroid hormone could not be thyroglobulin, but rather a breakdown product of this protein.

When, in the course of evolution, the thyroid gland severed its connection with the digestive system and the gland split into a large number of follicles, there arose a need for a different mechanism of hormone release. Available evidence indicates that there is still a digestion of the thyroglobulin present in the colloid of thyroid follicles, but this takes place *in situ*.

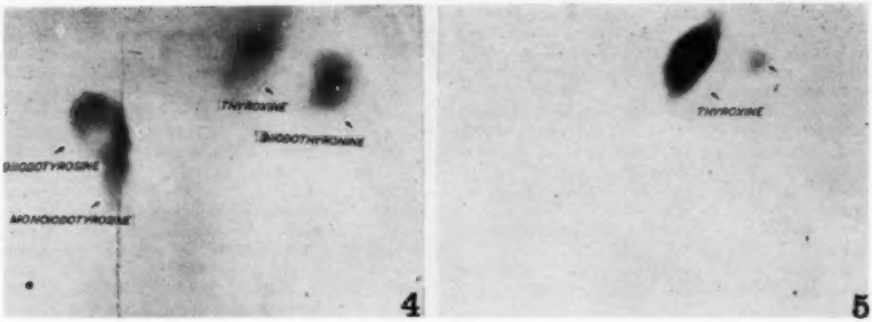
In 1941 de Robertis was able to suck the colloid out of a follicle of the thyroid isthmus in rats by means of a micropipette. When he placed a droplet of such colloid on solid gelatin, digestion of the gelatin ensued (72). Later, chemical estimation revealed the presence in the gland of a large amount of proteolytic enzyme capable of splitting tyrosine from proteins (73, 74). Furthermore, stimulation with pituitary extract increased, and hypophysectomy decreased the enzyme activity of the thyroid (74), thus indicating that this activity was under hypophyseal control. Recently, the presence of peptidase in the gland was also noted (75). de Robertis interpreted these results as demonstrating that proteolysis transformed the large molecule of thyroglobulin into smaller ones—which he described as polypeptides or small proteins—capable of diffusing out of the follicle through the cell wall and entering the circulation as thyroid hormone. Today it appears that his results can be more precisely interpreted. The proteolytic enzyme of the colloid, which was found to be capable of releasing tyrosine from proteins, might also presumably be capable of releasing the diiodotyrosine and thyroxin from thyroglobulin. If this were the case, free thyroxin would be present in the thyroid. The classical belief that thyroxin is not present as such in the gland (50) may have been due to the fact that the amount of this substance present was too small for chemical identification. Two recent techniques, the isotope dilution and the radioautography of paper chromatograms, were used to examine this possibility.

The isotope dilution method consists of mixing a minute amount of a radioactive substance such as thyroxin—which, according to this hypothesis, would appear in the thyroid gland after administration of radioiodine—with large amounts of a nonradioactive form of the same substance. In practice, a large amount of commercially available thyroxin was added to the freshly ground thyroid gland of a radioiodide-treated animal. This thyroxin should mix with the free radiothyroxin present and thus carry it through precipitation or crystallization procedures. If, after several precipitations, the thyroxin no longer contained radioactivity, then it would be possible to conclude that no free thyroxin was present in the gland. On the other hand, a definite proof of the presence of free thyroxin would require that the precipitate contains radioactivity after several different types of precipitation or crystallization, and also that the amount of radioactivity in the precipitate does not change, as shown by a constancy of the radioactive counts per microgram of substance (or specific activity).

This method was applied to the thyroid of adult rats sacrificed at seventeen hours after administration of radioiodine. After an initial fall, the specific activity of thyroxin remained constant through nine successive recrystallizations. Thus it was proved that there was a demonstrable moiety of radioactive thyroxin. Under the conditions of the experiment (76), approximately 1% of the total iodine in the gland was in this "free" thyroxin form.

A possible criticism of this technique was that the radioactivity might not have been due to thyroxin itself but to a closely related compound which had similar solubilities. The method of paper chromatography offered the possibility of a more precise identification of the thyroxin or thyroxin-like substance(s) present in the thyroid gland. A method was devised by which thyroxin, diiodotyrosine, monoiodotyrosine, and iodine could be separated chromatographically (Figs. 4 and 5). For this the thyroid glands of rats previously given radioiodine were rapidly ground in the cold and extracted with butanol. This butanol extract was then chromatographed on paper and the paper subsequently exposed to X-ray film. If free thyroxin had formed in the thyroid from the radioiodine administered, radioactivity should be present in the areas of the paper where the thyroxin was located. The application of this method revealed that not only free thyroxin but free diiodo- and monoiodotyrosine also were present in the thyroid gland. It was concluded from these results that all the iodinated amino acids known to exist in thyroglobulin could be found free in the thyroid gland. Presumably these substances must have been released from thyroglobulin by the enzymatic rupture of their peptide bonds (3, 6).

An important result of proteolysis should be considered here. The colloid of the follicle is contained in a closed space into which new molecules of thyroglobulin are continuously entering. The total number of molecules is further increased by the breakdown of each thyroglobulin molecule into a large number of amino acids. If osmotic pressure in a follicle is to be maintained at a constant level, a number of molecules equal to that appearing in the colloid over a given period of time must be removed from the



Figs. 4 and 5.—Comparison between a paper chromatogram (Fig. 4) and its radioautograph (Fig. 5). Plasma of rat sacrificed forty-eight hours after injection of radioiodine and extracted with butanol. To the butanol extract 50 μg. of monoiodotyrosine, diiodotyrosine, thyroxin, and diiodothyronine each were added. All that was deposited on the lower left-hand corner of the paper and chromatographed along both dimensions. The paper was dried, exposed to an X-ray film for one week, and the film was developed and fixed. The paper was then sprayed with diazotized sulfanilic acid to show the location of the four carriers as may be seen in the left picture (Fig. 4).

The X-ray film visible on the right (Fig. 5) shows an intense reaction due to radioactivity present in a location corresponding exactly to that of the thyroxin spot of the paper (Fig. 4); hence the conclusion that radiothyroxin was in this plasma. A spot to the right of the thyroxin—here labeled 1—(Fig. 5) is not located in exactly the same area as the contiguous diiodothyronine spot of the paper (Fig. 5). It is due to an unknown substance referred to as compound 1. (Gross, J., and Leblond, C. P., unpublished.)

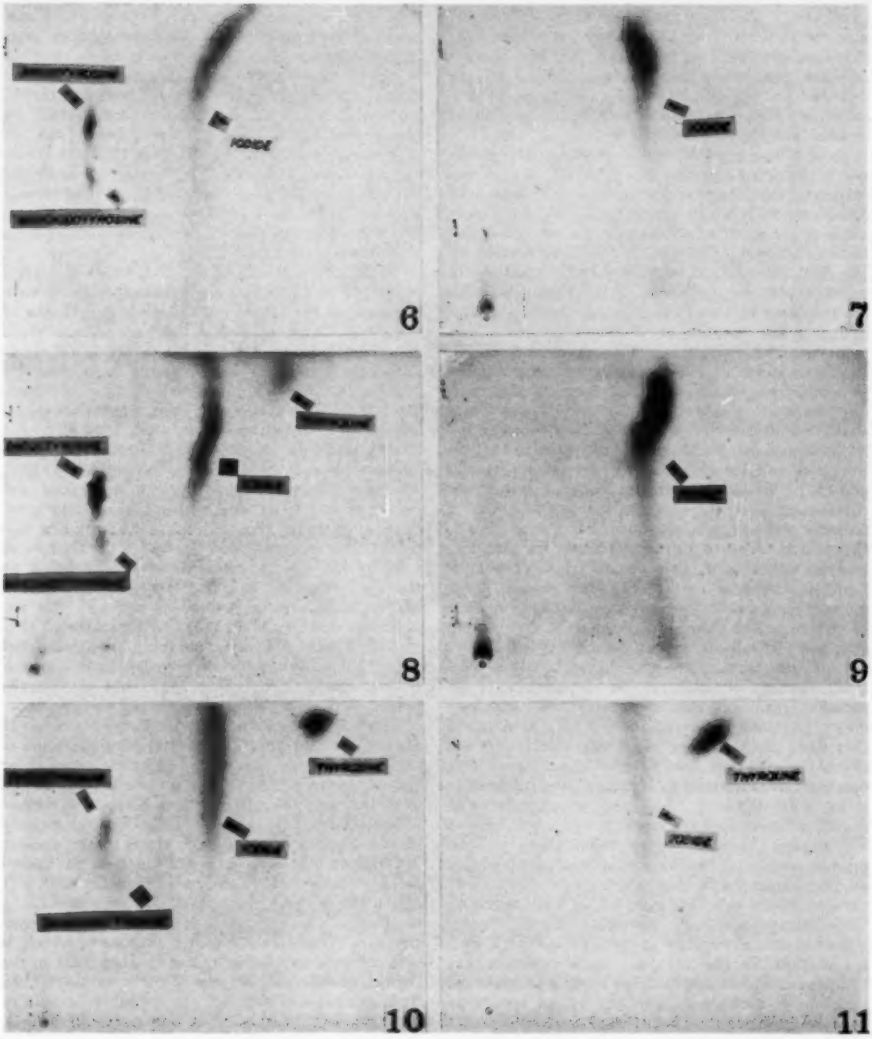
colloid. Since molecules of amino acids are more readily diffusible than those of larger proteins, such as thyroglobulin, these amino acid molecules should leave the colloid far more readily than thyroglobulin. This indeed they appear to do. Their eventual fate will be examined below.

By immunological techniques, Lerman showed that there was no thyroglobulin in peripheral blood, or even in the blood of the thyroid vein (77). On the other hand, the organic iodine of the blood was precipitable with trichloracetic acid and other protein precipitants, and, like a protein, was not dialyzable (78, 79). Hence, the consensus over the last few years has been that the circulating thyroid hormone was in the form of a small protein or polypeptide. It was referred to as the "protein-bound iodine" of plasma and was believed to be closely associated with the plasma albumin fraction of blood. In 1939, however, Treverrow did *in vitro* experiments which suggested that the organic iodine of the blood might consist of diiodotyrosine and thyroxin (80). Later, Harington supplied evidence of an indirect nature pointing to thyroxin as being the circulating hormone (81). Recently Taurog and Chaikoff (82) applied the isotope dilution technique to plasma of radioiodine-injected rats and directly demonstrated that thyroxin itself was the iodinated moiety of the so-called "protein-bound iodine." This important finding was independently confirmed with Gross in this Laboratory in a paper which also showed that the concentration of free thyroxin in the thyroid was much greater than in the plasma (76). The presence of thyroxin in blood was finally confirmed by paper chromatography (3, 6, 83–85; see also Figs. 4 and 5).

In addition to thyroxin, the blood contained iodide and the substance referred to above as compound 1. Diiodotyrosine was never demonstrable in blood, no matter at what time interval after injection of radioiodine the animals were sacrificed. Thus, the widely held belief arising out of Treverrow's work that this substance is present in blood was not substantiated.

Experiments in which radioautographs of chromatograms were prepared from butanol extracts of thyroids and plasma at various time intervals after radioiodine injection showed the sequence of events leading to the passage of thyroxin into the circulation. At fifteen minutes after injection, only radioiodide and radiothyroglobulin were present in the thyroid, while only radioiodide was detectable in plasma. Obviously, the circulating radioiodide had entered the thyroid gland and some radiothyroglobulin had been synthesized. At one hour after injection, free thyroxin was detectable in the thyroid, but not in blood. At three hours, thyroxin had appeared in blood also, where it became abundant by seventeen hours (Figs. 6–11). Thus, the sequence of events corresponds to the concept that thyroglobulin gives rise to thyroxin in the thyroid gland, and that later this thyroxin is excreted into the blood (3).

Other substances than thyroxin, namely, diiodo- and monoiodotyrosine, as well as several unidentified substances, may be recognized on paper chromatograms of the thyroid. Of all of these, apparently only thyroxin and possibly compound 1 are capable of leaving the colloid of the thyroid follicle and entering the circulation. The possibility still remains that some of these substances, such as diiodotyrosine, may leave the gland, but are so rapidly metabolized (8), that they cannot be detected even on paper chromatograms. Theoretically, it is far more likely that all these substances are metabolized within the gland. In this regard there are two possibilities. Diiodotyrosine may be broken down to iodide, a fact supported by the finding that radiodiiodotyrosine is broken down rapidly when placed in contact with thyroid slices, whereas thyroxin is broken down very slowly under similar conditions (86). Another possibility, which does not exclude the pre-



Radioautographs of paper chromatograms of the butanol extracts of unhydrolyzed thyroids (Figs. 6, 8, and 10) and plasmas (Figs. 7, 9, and 11) of rats receiving an adequate iodine intake (Purina Fox Chow). The animals received a subcutaneous injection of 100 μc. of carrier-free radioiodine. All the radioautographs were given the same exposure. (Gross and Leblond, reference 3.)

Fig. 6.—Thyroid extract of animal sacrificed one hour after radioiodide injection. Iodide, diiodotyrosine, and monoiodotyrosine spots are identified.

Fig. 7.—Plasma extract of animal sacrificed one hour after radioiodide injection. Iodide is the only definite spot.

Fig. 8.—Thyroid extract of animal sacrificed three hours after radioiodide injection. A clear-cut thyroxin spot has appeared.

Fig. 9.—Plasma extract of animal sacrificed three hours after radioiodide injection. Iodide is the only definite spot.

Fig. 10.—Thyroid extract of animal sacrificed twenty-four hours after radioiodide injection. The thyroxin spot is now fairly intense.

Fig. 11.—Plasma extract of animal sacrificed twenty-four hours after radioiodide injection. A clear-cut, intense thyroxin spot has appeared in addition to iodide.

vious one, is that diiodotyrosine, monoiodotyrosine, and the unknown iodinated substances are, like thyroxin, ejected from the colloid by the osmotic pressure, but, unlike thyroxin, are trapped by the cells for the building up of further thyroglobulin molecules, thus setting up an intra-follicular circulation of small iodinated moieties.

Some information is also available on the condition of the circulating thyroxin in blood. Since it was suspected that the thyroid hormone is in some way associated with blood proteins (87), attempts were made to see whether plasma proteins act as the carrier of thyroxin, employing the method devised by Franklin and Quastel, who used buffer solutions to mobilize proteins on paper. While plasma proteins are displaced by this method, thyroxin alone is not, and, therefore, remains at the origin. In an experiment carried out with this technique, a drop of plasma from a rat given radioactive iodine forty-eight hours previously was chromatographed and the paper was later radioautographed. In this way it was found that the radioactivity present in blood is associated with the plasma proteins, since the protein spot on the paper and the radioautograph coincide (Figs. 12 and 13). If radiothyroxin alone, however, is chromatographed in the same way with buffers, it remains at the origin (Fig. 14). But when radiothyroxin is added to a drop of plasma from an untreated animal and this plasma then chromatographed, thyroxin moves with the protein in a manner identical to that observed with the plasma of radioiodine-treated animals, that is, the protein spot and the radioautograph coincide (Figs. 15 and 16). Therefore, it can be stated that, by addition of small amounts of radiothyroxin to plasma, a combination results which duplicates that obtained under physiological conditions (6). Presumably, therefore, as the molecules of thyroxin leave the thyroid gland, they associate with proteins. This combination, although it is rather loose (it readily splits with butanol), is strong enough to carry thyroxin throughout the body.

Recently, this work was repeated using purified plasma proteins. Thyroxin readily associated with many plasma protein fractions, such as albumin and the globulins of the *alpha* type (88). Very recently, Gross, using paper electropheresis (*Nature*, in press) observed that thyroxin was mainly associated with α-1-globulin in the plasma. It is possible that thyroxin combines with a large variety of proteins, any one of which can then act as its carrier in organs and tissues. Thus, the passage of thyroxin through cell membranes could be effected by the migration of thyroxin from protein molecule to protein molecule.

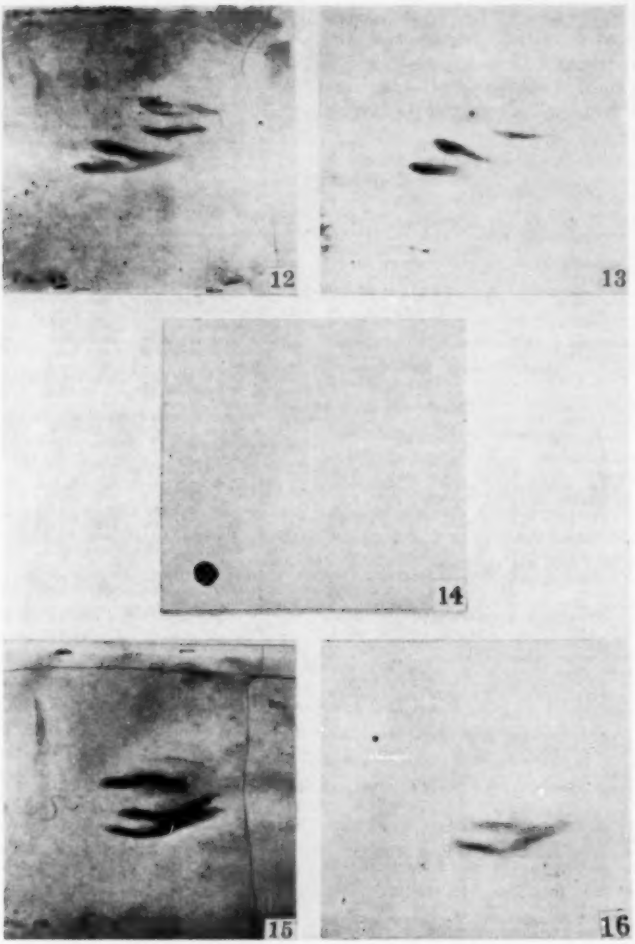
The Fate of Thyroxin in the Body.—The fate of thyroxin in the body was examined, using thyroxin labeled with radioiodine in the 3′,5′ positions. This was first carried out by Courrier and collaborators in rabbits. Little radioactivity was found in the thyroid, but significant amounts were present in the bile. The pituitary gland also appeared to fix more radiothyroxin than most of the other organs (89), a result recently attributed to an uptake by the posterior lobe of rabbits (90). When these experiments were repeated in rats, no special concentration was found in the hypophysis. The thyroid gland fixed little radioactivity, and that only after a sufficiently long time interval for a fair fraction of the administered radiothyroxin to have broken down and thus released its iodine. The outstanding phenomenon appeared to be the role played by the liver in the excretion of the material. Soon after injection, the liver content was very high, while later the material appeared in the intestine and feces. By twenty-four hours, up to 10% and more of the administered dose had been excreted in the feces, where the material was identified as thyroxin. A moderate amount of radioactivity was also found in urine and stomach in the form of iodide. In these locations, therefore, only the material (I^-) broken down from thyroxin is excreted (91).

While these results were obtained with large, nonphysiological quantities, recent experiments gave a picture of the fate of physiological doses using as little as 0.007 μg. of labeled thyroxin. Under these conditions the role of the liver in excreting the radioactivity through the bile into the feces was still prominent, although much less so than after administration of large doses. There was excretion into the urine of the iodide broken down from thyroxin. The rate of total excretion (fecal and urinary) was slower than with the large doses but still fairly rapid. The distribution of radiothyroxin in organs and tissues proved to be similar to that observed several days after radioiodide injection, i.e., after a sufficient time interval had elapsed for the thyroid to take up the injected radioiodide and release it as thyroid hormones. In other words, the distribution of the endogenous thyroid hormone in this experiment was like that obtained with labeled thyroxin. This result again confirms the fact that thyroxin is the thyroid hormone and that observations made with labeled thyroxin truly indicate the pathways of metabolism of this hormone (25).

The chemical nature of the iodinated substances arising in organs and tissues after administration of radiothyroxin was also investigated. One of these substances was compound 1. The occurrence of this substance in both thyroid and plasma of normal animals given injections of radioiodine has already been mentioned (3). Furthermore, in the present experiment this substance was found in thyroidectomized animals given radiothyroxin (19). It is, therefore, probable that this substance is a metabolite of thyroxin and can occur wherever thyroxin is present. Another substance, which is referred to as compound 4, was also observed in the organs, particularly in the kidneys of mice (19). This substance is abundant in the bile where no or only traces of thyroxin are found (unpublished). Since thyroxin is abundant in intestine and feces (19) and must go to the intestine through the bile (91), it is likely that compound 4 is a thyroxin complex which may regenerate thyroxin in the intestine. The eventual absorption of this thyroxin could give rise to an entero-hepatic circulation.

CONCLUSIONS

Two types of problems have hardly been touched in the field of endocrinology: the mechanism of hormonal elaboration by glands of interval secretion; and the intimate process by which hormones exert their peripheral effect. The first problem was clarified by the reported results, since many of the steps in the synthesis



Figs. 12–16, from Gross, Leblond, Franklin, and Quastel, reference 6.

Fig. 12.—A chromatogram of 0.02 ml. of the plasma of a rat given 100 μc. of carrier-free NaI^{131} subcutaneously forty-eight hours previously. A small amount of hemin has been added to the plasma. Since hemin is known to combine with proteins, the location of the proteins is indicated by staining of the hemin with benzidine. This appears as several elongated dark bands. The pencil lines indicate the leading edges of the developing solutions.

Fig. 13.—The radioautograph corresponding to the chromatogram making up Fig. 12. The radioactive material of the plasma is arranged in a pattern similar to that of the proteins.

Fig. 14.—The radioautograph of radioactive thyroxin, chromatographed with buffers as in Fig. 12. The radioactive thyroxin has not moved from the origin.

Fig. 15.—A chromatogram of 0.02 ml. of nonradioactive plasma, to which hemin and also radiothyroxin have been added, and subsequently chromatographed with buffers as in Fig. 12 and sprayed with benzidine. The protein pattern is apparent.

Fig. 16.—The radioautograph corresponding to the chromatogram making up Fig. 15. The radioactive thyroxin added to nonradioactive plasma has moved with the proteins, giving a picture similar to that obtained with radioactive plasma (see Figs. 12 and 13).

of the hormone, thyroxin, by the thyroid gland, have now been elucidated. Furthermore, the use of labeled thyroxin offers a promising approach to the study of the second problem: the mechanism of the peripheral effect of the thyroid hormone.

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The Determination of Alcohol in Ether by Infrared Absorption*

By A. A. COLON† and H. A. FREDIANI

Ethanol may be quantitatively determined in ether by measuring the absorbancy at 2.83 μ and making a correction for the absorbancy of the water present as measured by a Karl Fischer titration. The infrared absorption method presented here is more rapid and accurate than the chemical methods in current use. Once the calibration curve has been established, a determination can be carried out in fifteen minutes with a relative accuracy of ±2 per cent.

ETHYL ETHER is usually prepared commercially by the action of sulfuric acid on ethanol. As a result it usually contains a small percentage of the latter as an impurity. Ether that is to be used for anesthesia is prepared to contain 2 to 4 per cent of ethanol in order to decrease its volatility. The methods currently employed for the determination of ethanol in ether and in many other organic liquids make use of the oxidation of the alcohol to acetic acid with a known amount of an oxidizing agent and back titration of the excess oxidant (1–3). Other methods not in such common use involve absorption of the moisture by anhydrous potassium carbonate and of the alcohol by manganous chloride (4); absorption of the alcohol-containing ether in benzin and water with the distribution of the ether into the benzin and of the alcohol into the water layer. The increase in volume of each of these layers is used (5) for quantitative work. All of these methods are cumbersome and time-consuming. It was felt, therefore, that a method based on the strong and characteristic infrared absorption band of the hydroxyl group in the region of 2.83 μ might prove suitable for such quantitative estimations. The main, and practically only, source of interference would be water. However, it is relatively easy to determine the moisture present by means of a Karl Fischer titration and to apply the proper correction to the observed infrared absorbancy.

REAGENTS

A sample of absolute ether was treated to remove residual water and alcohol by drying with anhydrous calcium chloride, decanting, and then adding sodium ribbon. The ether was repeatedly decanted and fresh sodium ribbon added until all evolution of gas ceased. It was then filtered and distilled, keeping the system essentially anhydrous. This ether was then used as a reference standard and to prepare, for calibration purposes, solutions containing 0.1, 0.2, 0.3, and 0.5% of water and 0.5, 0.8, 1.0, 1.2, 1.5, and 2.0% of ethanol.

EXPERIMENTAL

A Beckman Spectrophotometer Model IR-2 equipped with 0.1-mm. rock salt cells was used throughout this work. The instrument was set for each series of determinations by using a 1% ethanol-in-ether solution and locating the absorption maximum manually between the values of 2.80 and 2.85 μ (Fig. 1). The wave-length setting of the instrument was not changed subsequently during any series of measurements. For calibration purposes the instrument was adjusted to read an arbitrary but convenient transmittancy (47%) using a clean wire gauze (18-mesh metallic screen). Using this reference standard the absorbancies of the previously prepared ether solutions containing known

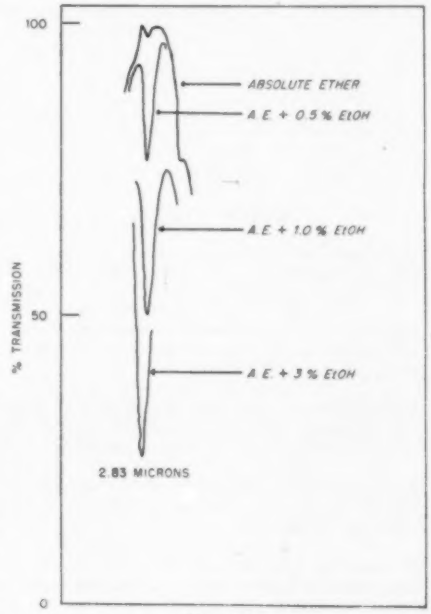

Figure 1

* Received June 19, 1951, from the Chemical Control Division, Merck & Co., Inc., Rahway, N. J.
† Present address: Puerto Rico Development Co., Puerto Rico.

TABLE I

Sample	H_2O (K-F), %	Dilution	A_W	A_T	A_C	EtOH, %	EtOH, % Original Sample
1—AR*	0.014	None	0.014	0.147	0.133	0.04	0.04
2—AR	0.004	None	0.004	0.276	0.272	0.44	0.44
3—AR	0.002	None	0.002	0.356	0.354	0.67	0.67
4—AR	0.002	None	0.002	0.261	0.259	0.40	0.40
5—R	0.17	1:1	0.083	0.557	0.474	1.02	2.04
6—R	0.28	1:3	0.068	0.558	0.490	1.07	4.28
7—R	0.75	1:1	0.365	0.866	0.501	1.10	2.20
8—U. S. P. X	0.17	1:1	0.083	0.726	0.643	1.50	3.00
9—U. S. P. X	0.17	1:1	0.083	0.717	0.634	1.48	2.96
10—U. S. P. X	0.16	1:1	0.078	0.681	0.603	1.39	2.78

* AR—Absolute Reagent Ether. R—Reagent Ether. U. S. P.—U. S. P. X ether.

amounts of water and ethanol were measured and calibration curves established as shown in Fig. 2. The absorbancy of the ether sample to be analyzed was then measured at 2.83 μ. A correction for the absorbancy due to water present was applied and the alcohol content then calculated. If the sample contained a relatively high amount of alcohol and thus had an absorbancy greater than 0.8, a quantitative dilution was first made, using the alcohol-free ether as diluent. The correction for its water content was applied, taking into consideration that it had been modified by the dilution.

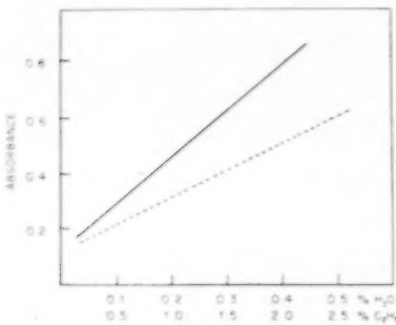

Fig. 2. —— alcohol, - - - - - water.

CALCULATIONS

The measured absorbancy (A_T) of the sample at 2.83 μ represents the sum of the absorbancies due to the ether (A_E), alcohol (A_A), and water (A_W):

$$A_T = A_E + A_A + A_W$$

Over a limited range of low concentrations of water and alcohol A_E may be considered as constant so that:

$$A_T = A_A + A_W + C$$

In the range 0 to 0.5% water in ether, the contribution by water to the total absorbancy at the indicated wave length is given by the expression:

$$A_W = 0.972 \times \% \text{ water}$$

By correcting the total absorbancy for the contribution due to water, the absorbancy due to ethanol and ether (A_C) is obtained:

$$A_A + C = A_C = A_T - A_W$$

By referring this value to the calibration curve prepared with known concentrations of ethanol in anhydrous ether, the amount of alcohol in the sample is readily obtained.

The numerical calculations are illustrated by a typical sample which was found to have a moisture content of 0.17%. Its absorbancy at 2.83 μ was much over 0.8 so a 1:1 dilution with the specially purified ether was made. The absorbancy then was found to be 0.726. The diluted sample contained only half of 0.17 or 0.085% of water. This contributed 0.972×0.085 or 0.083 to the absorbancy. The remaining absorbancy due to the alcohol and ether was $0.726 - 0.083$ or 0.643. By referring to the standard curve for ethanol, it was found to correspond to an alcohol content of 1.50%. Since this was for a 1:1 dilution, the alcohol content of the original sample was, therefore, 3.00%.

As a test for the validity of the method two solutions were prepared from the purified ether; one containing 0.2% water with 1.0% ethanol, the other 0.4% water with 2.0% ethanol. Because of its high absorbancy the latter sample was diluted 1:1 with specially purified ether. The alcohol contents were then determined spectrophotometrically with the following results:

Sample	H_2O, %	A_W	A_T	A_C	Ethanol Found, %
A	0.2	0.194	0.731	0.537	0.95
B	0.2	0.194	0.734	0.540	0.96

The results, 0.95% and $2 \times 0.96 = 1.92\%$, are in excellent agreement with the prepared compositions of the solutions.

To determine the reproducibility of the method, replicate determinations on a sample of reagent grade ether were made over a period of three days. In this work all measurements were made in a single cell to minimize variations in cell thickness and scattering. The following results were obtained:

	A_T
0.167	0.167
0.168	0.171
0.172	0.166
0.164	0.167
0.172	0.172
Av.	= 0.168
Av. deviation	= 0.0026 = 1.5%
Max. deviation	= 0.008 = 4.7%

Results on other samples are shown in Table I.

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Titration of Thiamine Salts with Perchloric Acid*

By CHARLES W. PIFER and ERNEST G. WOLLISH†

A method for the rapid assay of thiamine hydrochloride and mononitrate in commercial form by direct titration with perchloric acid is presented. The reaction is based upon the relative basicity of the thiamine moiety in a glacial acetic acid solvent. A precision of ±0.1 per cent has been obtained and the results agree within ±1.0 per cent with those obtained by the U. S. P. thiochrome method. Interference due to other compounds with basic groups can be compensated for by qualitative and limiting quantitative reactions, which are described.

A CONSIDERABLE number of fluorometric and colorimetric procedures for the determination of thiamine have been proposed. Most of the fluorometric methods depend in principle upon the oxidation of thiamine to thiochrome, while the colorimetric methods are based upon a reaction with diazotized aromatic amines. In Table I some of the methods more frequently used are shown, grouped according to the principle of the reaction involved.

While some of the fluorometric and colorimetric methods are very useful for the determination of vitamin B_1 in complex preparations, they are tedious and require sensitive measuring instruments. In addition, they suffer from the fact that standards must be run alongside the samples and the final measurement is carried out on only a few micrograms of material. In the case of the commercial bulk material, this requires considerable dilution and the results lack good precision.

A method for the direct titration of thiamine salts was therefore developed, based upon our previous work on titration of salts of organic bases in acetic acid (35). Literature references concerning this subject are listed in the bibliography of that publication.

THEORETICAL

Thiamine hydrochloride possesses two groups of basic character, consisting of a primary amine in the pyrimidine ring, and a quaternary nitrogen, formed by the linkage of the pyrimidine and thiazole rings. Both of these basic groups are susceptible to titration with perchloric acid.

Based on this reaction, a mechanism for the oxidation of thiamine to thiochrome in acid medium is postulated. If perchloric acid is added to thiamine hydrochloride in glacial acetic acid medium, no reaction occurs. If, however, the halogen ions are replaced with acetate ions at room temperature by the addition of mercuric acetate to a solution of thiamine hydrochloride, the titration with perchloric acid proceeds stoichiometrically. In this case, the resulting solution does not show fluorescence. When the same reaction is carried out in a hot solution, a definite fluorescence becomes apparent, having the same absorption spectrum as thiochrome with a maximum of 375 mμ.

TABLE I.—METHODS PROPOSED FOR THE DETERMINATION OF THIAMINE

Oxidizing Agent	References
Fluorometric Thiochrome Methods	
Potassium ferricyanide[a]	(2), (5), (6), (7), (10), (11), (12), (15), (16), (17), (19), (21), (27), (32), (33), (34), (39), (40), (43), (44), (45), (46)
Hydrogen peroxide Selenium dioxide[a]	(26)
Potassium permanganate Manganese dioxide[a]	(22)
Ceric sulfate	(9)
Quantitative Colorimetric Methods	
Reagent	
Diazotized amines	(25)
Diazotized sulfanilic acid	(23), (30)
Diazotized 2,4-dichloroaniline	(48)
Diazotized *p*-nitroaniline	(28)
Diazotized ethyl-*p*-aminobenzoate	(24)
Diazotized *p*-aminoacetophenone	(2), (3), (4), (8), (13), (18), (29), (36), (37), (40)
Qualitative Colorimetric Methods	
2,4-Dichlorobenzenediazonium chloride	(47)
p-Dimethylaminobenzaldehyde	(42)
Alcoholic 2,6-dibromoquinonechlorimide	(38)
Potassium bismuth iodide	(31)
Hydrosulfite	(27)
Phosphotungstate reduced with nascent hydrogen	(1), (41)
Ferric chloride and potassium ferricyanide	(20)
p-Aminophenylketone *p*-Aminoacetanilid	(37)
Others	
Sodium azide	(14)

[a] *p*H of reaction—alkaline.

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† The authors gratefully acknowledge the helpful suggestions of E. G. E. Shafer. Charles Isack carried out many of the determinations. Morton Schmall developed the colorimetric procedure for the determination of 6-amino-5-aminomethyl-2-methylpyrimidine, based on work by H. M. Wuest and M. M. Creighton. The authors are indebted to W. J. Mader of Merck & Co. for samples of 2-methyl-5-ethoxymethyl-6-aminopyrimidine and 4-methyl-5-β-(hydroxyethyl) thiazole.

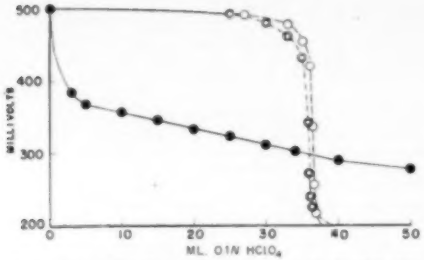

Fig. 1.—Titration of thiamine hydrochloride under varied conditions. Solid circle, no titration end point; double circle, mercuric acetate added cold (no fluorescence); open circle, mercuric acetate added hot (fluorescence).

Potentiometric titration curves are presented in Fig. 1, illustrating the various inflections obtained on individual samples under the conditions described. A concept of the mechanism of these reactions is suggested in Figs. 2, 3, and 4.

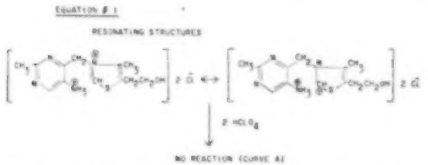

Fig. 2.—Attempted titration of thiamine hydrochloride with perchloric acid.

METHOD

Apparatus.—Fisher Senior Titrimeter, Beckman *p*H meter, using millivolt scale, or equivalent potentiometric instrument; Beckman Glass Electrode No. 1190-90; Beckman Calomel Electrode No. 1170.

Calomel electrodes were observed to be easily contaminated by mercuric acetate in glacial acetic acid, causing fluctuations of the galvanometer needle. If this condition occurs, the saturated KCl solution inside the electrode should be removed, the cell should be flushed with distilled water, rinsed with saturated KCl solution, and refilled with saturated KCl solution.

Reagents.—(*a*) 0.1 *N* Perchloric acid, prepared by mixing approximately 8.4 cc. of 70–72% perchloric acid with sufficient dioxane to make 1,000 cc.

This solution is standardized against potassium acid phthalate N. B. S. in the same manner as described for the titration of the sample.

(*b*) Glacial acetic acid, C. P.

(*c*) Mercuric acetate reagent, 6% in glacial acetic acid, dissolved hot and cooled to room temperature.

(*d*) Crystal violet indicator, 0.1% in glacial acetic acid.

Procedure.—A sample of appropriate weight (approx. 600 mg.), so as to consume approximately 35–40 cc. 0.1 *N* perchloric acid, is accurately weighed into a 250-cc. beaker and 80 cc. of glacial acetic acid is added. In the case of thiamine hydrochlo-

Fig. 3.—Titration of thiamine hydrochloride with perchloric acid after treatment with mercuric acetate in cold solution.

TABLE II.—COMPARISON OF PERCHLORIC ACID METHOD TO THIOCHROME METHOD OF THIAMINE HYDROCHLORIDE

Sample No.	Moisture, %	Perchloric Acid (Dry Basis), %	Thiochrome (Dry Basis), %	Deviation from Thiochrome, %
1	2.8	99.4	99.0	+0.4
2	3.2	99.8	100.5	−0.7
3	2.7	99.8	99.0	+0.8
4	3.5	99.9	100.2	−0.3
5	3.4	99.9	99.0	+0.9
6	3.1	99.8	99.7	+0.1
7	3.3	100.2	100.5	−0.3
8	2.8	99.8	99.7	+0.1
9	0.8	99.7	100.0	−0.3
10	2.1	99.3	100.3	−0.1
11	1.3	98.6	98.6	±0.0
12	0.9	98.8	98.8	±0.0

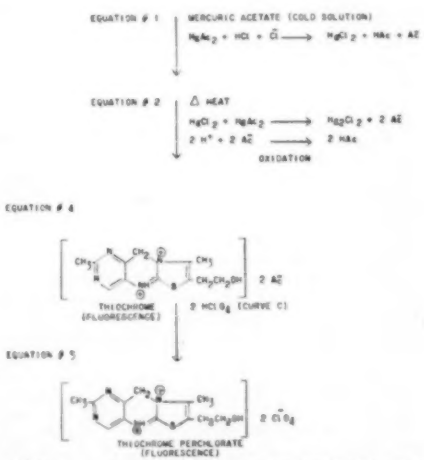

Fig. 4.—Titration of thiamine hydrochloride with perchloric acid after treatment with mercuric acetate in hot solution.

TABLE III.—PRECISION OF PERCHLORIC ACID METHOD

Titration No.	Thiamine Hydrochloride Potentiometric Titration %	Deviation from Mean	Thiamine Hydrochloride Visual Titration %	Deviation from Mean	Thiamine Mononitrate Visual Titration %	Deviation from Mean
1	99.7	±0.0	99.6	±0.0	99.1	±0.0
2	99.6	−0.1	99.7	+0.1	99.0	−0.1
3	99.7	±0.0	99.7	+0.1	99.2	+0.1
4	99.6	−0.1	99.6	±0.0	99.2	+0.1
5	99.7	±0.0	99.6	±0.0	99.1	±0.0
Mean:	99.7%		Mean:	99.6%	Mean:	99.1%
Av. Deviation:	±0.1%		Av. Deviation:	±0.1%	Av. Deviation:	±0.1%

ride the mixture is warmed to obtain complete solution, then cooled to room temperature and 10 cc. of mercuric acetate reagent is added. (Thiamine mononitrate does not require heating or addition of mercuric acetate.) The solution is titrated either potentiometrically or visually using 0.5 cc. of crystal violet indicator solution.

In the potentiometric titration the millivolts are recorded every 0.1 cc. in the vicinity of the calculated end point. From the values obtained, the exact end point is determined by extrapolation of a graph, plotting $\frac{\Delta E}{\Delta V}$ *vs.* V (cc.). Two equivalents of perchloric acid are consumed per mole of thiamine (Figs. 5, 6).

If a visual titration is desired, the solution is titrated to the same color-shade at the end point, as previously determined by visual standardization of the perchloric acid against potassium acid phthalate using the same amount of indicator solution.

Visual Titration with 0.01 *N* Perchloric Acid.—For the visual titration of small quantities of thiamine salts using 0.01 *N* perchloric acid, the following modified procedure should be followed, since the macro procedure would result in an indistinct end point.

A sample of thiamine hydrochloride (50–60 mg.), so as to result in a consumption of 30–35 cc. 0.01 *N* perchloric acid, is dissolved by warming with 5–10 ml. glacial acetic acid. After cooling to room temperature, 1 cc. of mercuric acetate reagent, 70–80 cc. of benzene, and 2–3 drops of crystal violet indicator are added in the sequence indicated. The solution is titrated with 0.01 *N* $HClO_4$ in dioxane to the same color-shade as obtained in the visual standardization of the titrant, carried out under the same conditions.

Results.—In Table II the titration results with perchloric acid on 12 samples of U. S. P. thiamine hydrochloride are compared against those obtained by the U. S. P. thiochrome method (both calculated on anhydrous basis). From the values recorded it is evident that the methods agree within about ±1.0%.

The precision of the perchloric acid titration of thiamine salts is demonstrated in Table III. Samples from identical lots of thiamine hydrochloride and mononitrate were titrated by the methods described and the results calculated on anhydrous basis. They indicate a reproducibility of ±0.1%.

DISCUSSION

Interferences.—The perchloric acid method of titration is not a specific reaction for thiamine. Several of the intermediate compounds encountered in the synthesis of thiamine salts tend to result in too high titration values if present in material quantities in the final product. These intermediates, like thiamine itself, possess basic nitrogen groups, which are titrated with perchloric acid. However, their presence can be either qualitatively or quantitatively detected and their interference can thus be compensated for.

A correction can be applied for the presence of the following intermediates, which, if found, would be present as the hydrochloride or nitrate:

(1) 2-methyl-5-ethoxymethyl-6-aminopyrimidine, m. w. 167.2.

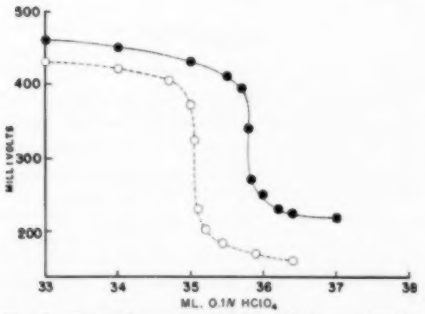

Fig. 5.—Potentiometric titration of ●— thiamine hydrochloride, ○- - - thiamine mononitrate.

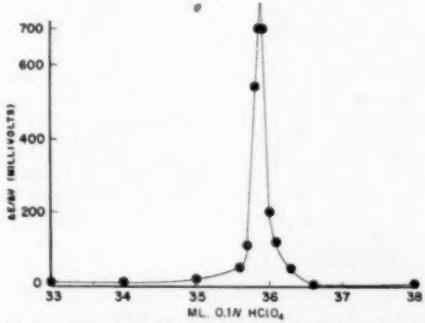

Fig. 6.—Titration of thiamine hydrochloride $\Delta E/\Delta V$ *vs.* V.

(2) 4-methyl-5-β-(hydroxyethyl)-thiazole, m. w. 143.2.

(3) 2-methyl-5-bromomethyl-6-aminopyrimidine, m. w. 202.1.

These basic compounds can be separated from thiamine by extraction with ether from alkaline solution. The ether extracts are evaporated, the bases dissolved in glacial acetic acid and titrated with perchloric acid. The amount of 0.1 *N* perchloric acid consumed is then subtracted from the total quantity of perchloric acid consumed by the same quantity of thiamine. Thus a correction can be applied in case of the presence of the compounds mentioned previously.

(4) 6-amino-5-aminomethyl-2-methylpyrimidine (subsequently referred to as compound 4), m. w. 182.3.

When this compound is condensed with ninhydrin (49), a reddish color is produced. This color, with a maximum absorbancy at 560 mμ, follows Beer's law within the specified concentration and thus can be utilized for a photometric procedure:

Apparatus.—Photoelectric colorimeter or spectrophotometer.

Reagents.—(*a*) Standard compound 4 solution, an aqueous solution of pure compound 4 dihydrochloride, m. p. 260° (corr.) in a concentration 60 μg./cc.

(*b*) Pyridine solution, pyridine C. P. diluted 1:1 with water.

(*c*) Ninhydrin reagent, a 1% aqueous solution of triketohydrindene hydrate. *Example:* Determination of compound 4 in thiamine hydrochloride.

Procedure.—An aqueous "blank" solution is prepared from dried U. S. P. reference standard thiamine hydrochloride, 25 mg./cc. The sample solution is prepared in the same concentration. Into each of four 25-cc. glass-stoppered graduates, marked "A," "B," "C," and "D," standard, samples, and reagents are added as follows:

	Cc.			
Graduate	A	B	C	D
U. S. P. ref. std. soln.	1.0	...	...	...
B_1 Sample solution	...	1.0	1.0	1.0
Distilled water	2.0	2.0	1.0	...
Compound 4 std. soln.	...	...	1.0	2.0
50% Pyridine solution	1.0	1.0	1.0	1.0
Ninhydrin reagent	1.0	1.0	1.0	1.0

Upon addition of all reagents, the graduates are immersed in a *boiling* water bath for fifteen minutes, cooled with an electric fan and then immersed in a water bath at about 25° for ten minutes, so as to attain room temperature. All graduates are then filled to the 25-cc. mark with distilled water. The photoelectric colorimeter, equipped with a filter of median wave length at 560 mμ, is set at zero absorbancy with the "blank" solution in graduate "A," and the absorbancy of the liquids in the remaining graduates is determined, resulting in readings for "B," "C," and "D," which are used in the following calculation:

Calculation.—

$$\frac{B \times \mu\text{g. of Compound 4 Std. in } (C + D) \times 100}{[(C + D) - 2B] \times 25{,}000^{1}} = \%\text{ compound 4 dihydrochloride}$$

[1] Dilution factor.

A correction for the amount of compound 4 found can be applied to the results obtained in the perchloric acid titration by using the following calculation:

$$\frac{A \times B}{C} = \%\text{ apparent thiamine hydrochloride.}$$

where

A = 0.33726 (1/1,000 molecular weight of thiamine dihydrochloride)

B = % compound 4 dihydrochloride found

C = 0.2553 (1/1,000 molecular weight of compound 4 dihydrochloride).

This figure should be subtracted from the total value obtained in the perchloric acid titration.

(5) 6-amino-5-thioformamido-methyl-2-methylpyrimidine, m. w. 182.3.

(6) sodium dithioformate, m. w. 100.1.

The presence of these compounds can be detected by heating an acidified solution and testing the vapors with moistened lead acetate paper for free hydrogen sulfide. They can be determined quantitatively by titration with 0.1 *N* iodine in acid solution.

SUMMARY

The direct titration method with perchloric acid in nonaqueous medium has distinct advantages over heretofore-used fluorometric and colorimetric methods for the assay of thiamine salts in commercial form. The method is simple and rapid and provides stoichiometric results without the use of complicated and costly instruments. It eliminates the necessity of running parallel determinations on standards, such as required for fluorometric and colorimetric measurements. The method has a high degree of precision and the results are comparable to those obtained by the U. S. P. thiochrome method. Although not specific for the thiamine molecule, interference due to other compounds with basic groups, encountered in the thiamine syntheses, can be compensated for by qualitative and limiting quantitative tests.

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Use of Paper Chromatography for the Quantitative Estimation of Quercetin in Rutin*

By J. NAGHSKI,† C. S. FENSKE, Jr., and J. F. COUCH‡

The rapid mobility of quercetin during chromatography on paper forms the basis of its separation and isolation from rutin preparations. The quercetin in the eluate is determined spectrophotometrically after reaction with aluminum chloride. The developing solvent can be modified to prevent interference by related flavonol compounds.

RUTIN (3,5,7,3′,4′-pentahydroxyflavone-3-rutinoside), unless purified by special techniques, contains varying amounts of quercetin (3,5,7,3′,4′-pentahydroxyflavone). Undoubtedly, some of the quercetin is formed by the hydrolysis of the glycoside during processing and some is extracted from the plant. Since quercetin probably has the same physiological activity as rutin, it would not be objectionable, yet its content does reflect the care exercised in the preparation of rutin. The National Formulary (1) specifies a 5 per cent limit on the quercetin content of rutin preparations. The similarity in structure of the two compounds makes it difficult to determine one in the presence of the other.

In 1947 Porter, Brice, Copley, and Couch (8) reported a spectrophotometric method for the simultaneous determination of rutin and quercetin; in 1949 a modification of the method was proposed by Swann (10). Both procedures require specialized apparatus and unusual care in standardization of wave length and preparation of sample, and both are subject to interference from related and extraneous impurities. Porter, Dickel, and Couch (9) developed a method for determination of small quantities of rutin in urine based on production of color with aluminum chloride. Later Dechene (5) adapted this reaction to determination of rutin in tablets. Neither of these procedures is specific for rutin, since other flavonols give the same reaction.

The development by Consden, Gordon, and Martin (3) of partition on a filter paper support gave a powerful tool for the fractionation of difficult mixtures. Since then the method has been applied not only to the separation but also to the quantitative determination of an ever increasing number of compounds. Use of paper chromatography for the separation and identifica-

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† The authors thank A. Turner, Jr., for the quercetin analyses by the ultraviolet spectrophotometric method and for determination of the spectral absorption curves.
‡ Deceased.

tion of flavonoid pigments has been reported by Bate-Smith and Westall (2) and by Wender and co-workers (6, 11). For a quantitative determination, Gage and Wender (6) eluted the flavonol-3-glycosides from the chromatograms with aluminum chloride and measured the intensity of the resulting colored complex with a spectrophotometer. The aglycone, quercetin, however, could not be eluted quantitatively because of adsorption of the complex by the cellulose.

It is possible to approximate the quantity of many compounds by measuring the size and intensity of the spot. Quercetin, however, tails excessively, making it impossible to judge the size or intensity of the spot with any degree of accuracy.

The technique described here depends on the fact that when the chromatograms are developed with ethyl acetate saturated with water, rutin moves slowly (R_f, 0.05), whereas quercetin travels rapidly (R_f, 0.90). By using a short strip of paper, the quercetin runs completely off the bottom while the rutin remains on the paper strip. The quercetin is determined by an adaptation of the method of Porter, Dickel, and Couch (9).

EXPERIMENTAL

Reagents

(*a*) Chromatographic solvents: either (*a*) ethyl acetate saturated with water; or (*b*) a mixture of ethyl acetate, benzene, and water (35:15:50).

(*b*) Aluminum chloride, 0.1 *M* aqueous solution.

(*c*) Potassium acetate, 1 *N* aqueous solution.

(*d*) Methanol or isopropanol.

Apparatus

(*a*) Filter paper, Whatman No. 1[1] cut as illustrated in Fig. 1.

(*b*) Micropipette graduated in 0.01 ml. and fitted with a 26-gauge hypodermic needle.

(*c*) Receivers, 6-oz. bottles cut off as illustrated in Fig. 1.

(*d*) Tank, a 10-in. desiccator fitted with a tray and receivers as shown in Fig. 2. A Pyrex desiccator without a continuous shelf can be adapted by using a perforated plate and a shorter pan support.

(*e*) Spectrophotometer or filter photometer with cell compartment for 2.5-cm. cells.

PROCEDURE

Standard Rutin Preparation.—Rutin was made free of contaminating flavonoid pigments by repeated recrystallization alternately from absolute (98–100%) ethanol and water (4).

Standard Quercetin Preparation.—The purified rutin was hydrolyzed by refluxing for two hours in 2% sulfuric acid. The quercetin was further purified by recrystallization from 70–80% ethanol (7). The spectral absorption curves for the quercetin-aluminum complex and pure quercetin are shown in Fig. 3. The absorptivity (a) = A/bc, where A is the absorbance (optical density) of solution of depth, b cm. and concentration, c grams per liter minus the absorbance of an equal depth of solvent.

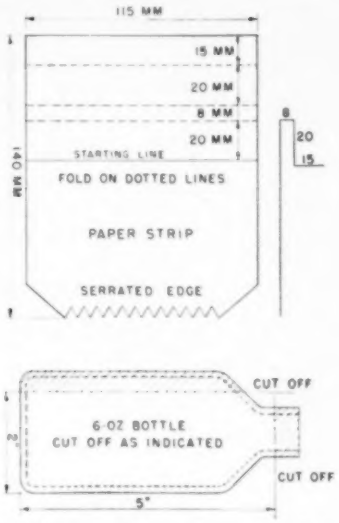

Fig. 1.—Diagram of paper strips and receivers used for the chromatographic elution of quercetin.

Separation and Isolation of Quercetin from Rutin.—A 1-Gm. sample of rutin is dissolved in 50 ml. of either methanol or isopropanol. With a micropipette, 0.05 ml. of this solution is applied to the starting line of the filter paper and distributed so that six or seven discrete spots of approximately equal concentration are obtained. By applying small droplets, the size of the spots is kept less than 1 cm. in diameter.

The filter papers are folded and suspended from the tray so that the serrated tips hang within the receivers. Since four pieces of paper can be accommodated at one time, it is possible to run the sample in triplicate with a plain sheet for a blank. The aqueous phase of the solvent is introduced into the

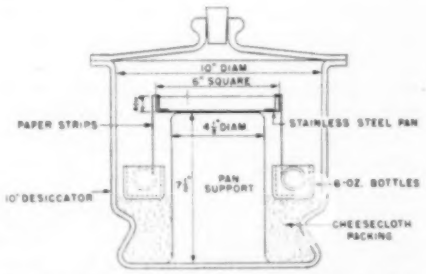

Fig. 2.—Arrangement of pan, paper strips, and receivers in chromatography apparatus.

[1] The mention of commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

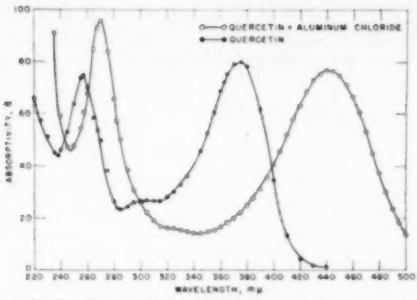

Fig. 3.—Spectral absorption curves for quercetin in 95% ethanol and for quercetin + aluminum chloride in potassium acetate buffered aqueous ethanol.

bottom of the desiccator, and the papers are allowed to equilibrate with the atmosphere for two to three hours. The water-saturated ethyl acetate is then added to the tray, and the chromatograms are developed for sixteen to eighteen hours. During this time, the rutin front moves about 2 to 3 in. down the chromatogram; the quercetin drips off the paper and appears in the eluate.

Spectrophotometric Determination.—The eluates containing the quercetin are transferred from the receivers to 100-ml. beakers by rinsing with alcohol. The solvent is evaporated on a steam bath. To each beaker is added 10 ml. of absolute alcohol, and 3 ml. of 0.1 *M* aluminum chloride solution. The resulting solutions are transferred to 25-ml. volumetric flasks, the beakers are rinsed with 5 ml. of 1 *N* potassium acetate solution, and the solutions are then made to volume with alcohol. After about twenty minutes and not later than one hour, the absorbance of each solution is determined at 440 mμ in a 2.5-cm. cell *versus* a solution in an equal cell prepared from a paper blank carried through the same procedure. After a longer time, the solutions become turbid because of formation of aluminum hydroxide. If excessive interference is caused by too rapid formation of turbidity, the amount of potassium acetate used can be reduced appreciably without affecting the determination, provided the same proportions of reagents are used in the preparation of a standard curve.

Preparation of a Standard Curve.—Pipette a volume of each standard solution, corresponding to 0.030, 0.045, 0.060, 0.075, 0.090, 0.105, 0.120, 0.135, and 0.150 mg. of quercetin per 10 ml. of alcohol, into 25-ml. volumetric flasks, add 3 ml. of 0.1 *M* $AlCl_3$ solution and 5 ml. of 1 *N* potassium acetate solution and make to volume with alcohol. The absorbance as measured against a reagent blank is plotted *versus* weight of aglycone taken for analysis. Table I shows the data obtained in the standardization. A straight line, passing through the origin, was obtained in the range of 0.03 to 0.15 mg. of quercetin.

Recovery Experiments.—In trials using quercetin alone, only 85–90% of the quercetin could be recovered. When rutin was present, however, the recovery was practically complete. This increase in recovery was undoubtedly due to displacement of quercetin by rutin during development of the chromatogram. Synthetic mixtures of rutin containing 4%, 5%, and 6% of quercetin were prepared and chromatographed. The recovery of the quercetin in the eluate is shown in Table II.

TABLE I.—STANDARDIZATION DATA. QUERCETIN-ALUMINUM COMPLEX

Wt. of Quercetin (C[a]), Mg.	Absorbance (A[b])	Absorptivity (a)
0.030	0.238	79.3
0.045	0.354	78.7
0.060	0.476	79.3
0.075	0.596	79.5
0.090	0.705	78.3
0.105	0.820	78.1
0.120	0.935	77.9
0.135	1.046	77.5
0.150	1.161	77.4

[a] C = mg. of quercetin in final 25 ml.
[b] A = absorbance at 440 mμ measured on a spectrophotometer in a 2.5-cm. cell with reference to an equal cell containing reagents.

TABLE II.—RECOVERY OF QUERCETIN FROM SYNTHETIC MIXTURES OF QUERCETIN AND RUTIN

Wt. of Quercetin Applied, Mg.	Wt. of Quercetin Determined, Mg.	Recovery, %
0.027	0.028	103
0.027	0.026	97
0.027	0.027	100
0.034	0.034	100
0.034	0.033	97
0.034	0.031	91
0.041	0.042	102
0.041	0.040	98
0.034[a]	0.031	91
0.034[a]	0.032	94
0.034[a]	0.032	94
		Av. 97

[a] Solvent composition: ethyl acetate, 35; benzene, 15; water, 50.

Detection and Elimination of Interfering Substances.—Rutin prepared from certain plants contains small amounts of flavonoid pigments in addition to quercetin. Since these substances usually migrate down the paper at a faster rate than rutin, they appear in the eluate and are determined as quercetin. It was found that the R_F values of flavonoids could be controlled by adding benzene to the ethyl acetate. The effect of different amounts of benzene on the R_F values of rutin, quercetin, and other flavonols is shown in Table III. Thus the addition of 15 parts of benzene was sufficient to inhibit the migration of the unknown flavonol glycosides without affecting appreciably the migration of

TABLE III.—EFFECT OF SOLVENT COMPOSITION ON R_F VALUES OF FLAVONOLS

	Solvent Composition, Ml.			
Ethyl Acetate	50	40	35	25
Benzene	0	10	15	25
Water	50	50	50	50
	R_F	R_F	R_F	R_F
Rutin	0.05	0.01	0.0	0.0
Flavonol A	0.32	0.20	0.05	0.0
Flavonol B	0.55	0.41	0.10	0.0
Quercetin	0.90	0.85	0.74	0.50

TABLE IV.—ANALYSIS OF RUTIN FOR QUERCETIN CONTENT BY CHROMATOGRAPHIC AND ULTRAVIOLET SPECTROPHOTOMETRIC METHODS

Sample	—Chromatographic, %—				—Ultraviolet Spectrophotometric, %—			
	1	2	3	Av.	1	2	3	Av.
A	3.3	3.4	3.4	3.4	2.6	3.0	2.6	2.7
B	4.1	4.1	4.2	4.1	4.6	3.9	3.9	4.1
C	5.8	5.8	5.6	5.7	5.9	6.9	...	6.4
D	8.5	8.7	8.8	8.7	9.2	9.4	...	9.3
E	5.2	5.0	5.1	5.1	6.3	5.7	...	6.0
F	2.3	2.6	2.6	2.5	0.0	−0.2	...	−0.1
	0.0[a]	0.0	0.01	Trace	...	...	...	...
G	5.6	5.8	5.7	5.7	4.3	4.0	...	4.2
	0.4[a]	0.3	....	0.4	...	...	...	...

[a] Modified solvent composition (ethyl acetate, 35; benzene, 15; water, 50).

quercetin. The use of this solvent did not interfere with the recovery of quercetin when admixed with rutin (Table II).

To detect the presence of the flavonol pigments which will interfere in the determination of quercetin, the rutin preparation is chromatographed by either the descending technique as recommended by Bate-Smith and Westall (2), or the ascending technique as recommended by Wender and Gage (11). The solution of rutin is applied to the starting line with a capillary pipette to give a spot about 1 cm. in diameter and containing 40 to 50 μg. of flavonol. The paper is equilibrated in an atmosphere of the aqueous phase for two hours and then developed with water-saturated ethyl acetate for four to six hours. The time required for the solvent to travel 30 to 40 cm. varies with the room temperature. After developing, the chromatograms are air-dried, sprayed with a solution of aluminum chloride, air-dried again, and examined under ultraviolet light.[3] The interfering flavonol pigments appear as fluorescent zones located between those of rutin and quercetin. This procedure is extremely sensitive, detecting as little as 0.2 μg. of quercetin. Spraying with potassium acetate as suggested by Dechene (5) quenches the fluorescence and reduces the sensitivity to 1 μg. of quercetin.

Application of Method to Rutin Preparations.—To establish the applicability of this method to the analysis of actual rutin samples, the results (Table IV) were compared with those obtained by the spectrophotometric method of Porter, Brice, Copley, and Couch (8).

DISCUSSION

Results obtained by the ultraviolet spectrophotometric method are in general higher and show greater variation than those obtained by the chromatographic procedure. The higher spectrophotometric values are undoubtedly due to the presence of interfering impurities. The rutin solutions were dark-colored (indicating the presence of absorbing pigments other than rutin and quercetin) and contained different amounts of materials insoluble in alcohol. Considering the inherent expected error by this method of ±0.7 in the percentage of both rutin and quercetin (8), the results obtained by the two procedures are in good agreement.

The chromatographic procedure gave high values with the two samples of buckwheat rutin (F and G). Chromatography of these samples revealed small proportions of flavonols other than rutin and quercetin. Two compounds could be detected with R_F values of 0.32 and 0.55 (Table III). (The characterization of these constituents will be the subject of a later paper.) When the modified solvent was used in the analysis of these samples, it was found that the true quercetin content was low. Thus in sample G the ultraviolet method apparently determined these constituents as quercetin, whereas with sample F an apparently reliable value was obtained, probably through a compensation of errors. The results point to the fact that the chromatographic procedure could be used not only to determine the quercetin but also the content of other minor flavonols in rutin preparations.

SUMMARY

A method has been developed for the specific determination of quercetin in rutin preparations. It is not subject to error due to interfering substances, and is not critical as to standardization of wave length and preparation of sample.

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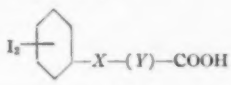
[3] Fluorescent black light tubes with purple envelope are satisfactory for this purpose.

The Preparation and Cholecystographic Properties of Some Aminotriiodophenylalkanoic Acids*

By S. ARCHER, J. O. HOPPE, and T. R. LEWIS

A series of β-(3-amino-2,4,6-triiodophenyl)-α-alkylpropionic acids was prepared starting with *m*-nitrobenzaldehyde and the required anhydrides. The α-alkyl *m*-nitrocinnamic acids so obtained were catalytically hydrogenated and the resulting amino acids then iodinated. The outstanding member of the series, α-ethyl-β-(3-amino-2,4,6-triiodophenyl)-propionic acid[1] gave more intense shadows than Priodax at all dose levels tested.

IN A PREVIOUS publication the minimum structural requirements of a good oral cholecystographic agent were discussed (1). Almost all of the compounds considered had two features in common, namely, a carboxyl group and two iodine atoms. The compounds can be represented by the general formula, I.



(I)

The hexagon represents either a carbocyclic or heterocyclic ring which usually carries an amino or hydroxyl group in addition to the two iodine atoms. The group, X, serves as a bridge linking the aromatic ring with the lipophilic group, Y, which in turn is commonly joined directly to the hydrophilic carboxyl group.

Recently Papa and co-workers (2) used quinolyl and pyridyl groups as the hydrophilic portion of the molecule. They observed that their compounds remained unabsorbed from the intestinal tract. It may be necessary to use stronger bases or water-soluble salts thereof to facilitate absorption. It is also possible that bases of smaller molecular weight should be used.

The activity of any cholecystographic agent will be proportional to that fraction of the total dose that appears in the gall bladder and to the iodine content of the drug. This may be expressed symbolically as in Eq. 1.

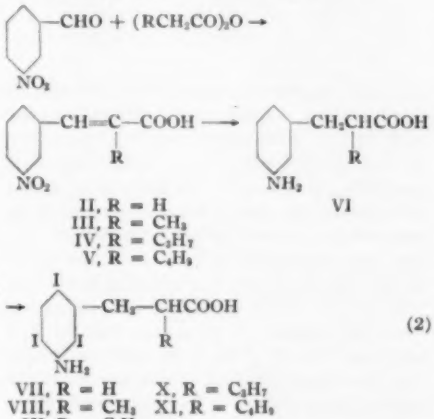
$$A = K\,(X/Y)\text{-per cent } I_2 \qquad (1)$$

Where A is the activity of the drug, X the amount present in the gall bladder, Y, the dose, and per cent I_2 is the iodine content.[2]

In our previous study (1) we pointed out that the minimum structural requirements of an orally effective cholecystographic agent are: (*a*) an aromatic ring to act as a halogen carrier, (*b*) a lipophilic group of moderate size to direct the drug to the gall bladder, and (*c*), a hydrophilic group or groups. When these groups are present in a diiodo compound the iodine content of the molecule is about 50 per cent (e. g., Priodax). If the ratio X/Y in Eq. 1 is unity then the activity of the drug will depend solely on the amount of iodine present. Thus we are confronted with the paradox that the very structures that are responsible for high activity actually set an upper limit on the activity obtainable.

If the structures that favor good activity in the diiodo class will also promote activity in the triiodo group then the limit will be raised since the latter group will then have iodine contents near 70 per cent. If the activity of Priodax is 100 and X/Y is unity then the maximum activity attainable in the triiodo series is 140.

With these considerations in mind we prepared and tested an homologous series of β-(3-amino-2,4,6-triiodophenyl)-alkanoic acids. One of these, IX, has been reported in another paper (3). The method of synthesis is shown in Eq. 2.



II, R = H
III, R = CH_3
IV, R = C_2H_7
V, R = C_4H_9

VI

VII, R = H
VIII, R = CH_3
IX, R = C_2H_5
X, R = C_3H_7
XI, R = C_4H_9

(2)

m-Nitrobenzaldehyde was condensed with the appropriate acids and acid anhydrides under the conditions of the Perkin reaction to furnish the *m*-nitrocinnamic acids, II–V. The acids II (4)

* Received July 6, 1951, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

[1] This substance has been given the trade name Telepaque.

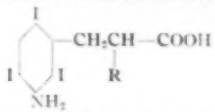
[2] The ratio X/Y can be measured experimentally. Preliminary investigations, to be reported at a later date, have been carried out by one of us (J. O. H.).

and III (5) were previously known. Both forms of IV were isolated from the reaction mixture. A low-melting acid, presumably the *cis* isomer, was separated during the purification of V, but in amounts too small to permit adequate characterization.

The nitro acids were reduced either as their sodium salts in aqueous solution or in ethanol as the free acids. In view of the fact that all the amino acids, VI, were difficult to obtain in pure form, they were iodinated without isolation with iodine monochloride in acid solution at elevated temperatures over long periods. Despite these drastic conditions halogenation was incomplete. Extensive purification was required to produce analytically pure specimens. Indeed in the case of X it was possible to isolate in pure form the corresponding diiodo acid.

Pharmacology.—The triiodo acids (VII–XI) were screened in cats at a dose level of 100 mg./Kg. as previously outlined (1). The activities are listed in Table I.

TABLE I.—CHOLECYSTOGRAPHIC ACTIVITIES OF THE AMINOTRIIODOPHENYLALKANOIC ACIDS



R =	Iodine (Calcd.), %	Activity (Priodax = 100)
H	70.1	56
CH_3	68.3	120
C_2H_5	66.7	144
C_3H_7	65.1	116
C_4H_9	63.6	16

It is of interest to compare this series with the corresponding 4-amino-3,5-diiodophenylalkanoic acids (3, 6). In the diiodo group maximum activity was reached when the side chain contained four carbon atoms. Here the four-carbon analog, XI, is practically devoid of activity (most of the compound was unabsorbed). The lower members were appreciably active. Even the unsubstituted acid, VII, was half as active as Priodax. The methyl (VIII) and ethyl (IX) derivatives were outstanding. If we assume that the X/Y ratio (*vide supra*) of IX is the same as Priodax then the theoretical value for the activity is 134, which is remarkably close to the observed value.

In a more intensive study of IX it was found that this drug gave more intense shadows than Priodax at all levels from 25 mg./Kg. to 200 mg./Kg. A more detailed pharmacological report on the series was presented elsewhere (7).

Our initial hope that the third iodine atom would not alter the structural requirements for cholecystography was fulfilled. The extra halogen appears to be equivalent to two methylene groups in the lipophilic portion of the molecule as judged by the occurrence of the maximum in the series. Further, the radiopacity was considerably increased.

EXPERIMENTAL[1]

β-(3-Amino-2,4,6-triiodophenyl)-propionic Acid.—A solution of 19.3 Gm. (0.1 m) of *m*-nitrocinnamic acid in 100 ml. of ethanol was reduced with the aid of Adams' platinum oxide. After seven and one-half hours, reduction did not seem to be complete. The catalyst was removed by filtration and the filtrate concentrated to dryness. The residue was taken up in 7% sodium hydroxide and stirred with 3% sodium amalgam for two hours. The solution was freed from mercury and concentrated to dryness. The residue was leached with alcohol. The leachings were concentrated and the residue dissolved in 50 ml. of 6 *N* hydrochloric acid. A solution of 16.8 ml. (0.33 m) of iodine monochloride in 50 ml. of 6 *N* hydrochloric acid was stirred at 70° while the solution of the amino acid was added. To this solution 300 ml. of water was dropped in over a two-hour period. The reaction mixture was cooled and filtered. The brown solid was dissolved in dilute sodium hydroxide and the dark solution then was treated with a small amount of saturated brine. On standing the sodium salt separated. It was removed by filtration and dissolved in water. The free acid was thrown out when sulfur dioxide was passed into the solution. The acid was collected on a filter and recrystallized from methanol. There was obtained 9.5 Gm. of material, m. p. 232.7 to 233.5° (corr.). The filtrate furnished 5.4 Gm. of product of comparable purity.

Anal.—Calcd. for $C_9H_{10}I_3NO_2$: I, 70.13. Found: I, 70.50.

β-(3-Amino-2,4,6-triiodophenyl)-α-methylpropionic Acid (VIII).—The reduction of 51.8 Gm. of α-methyl-*m*-nitrocinnamic acid was carried out in dilute sodium hydroxide in the presence of Raney nickel catalyst at 70–80°. After the theoretical amount of hydrogen was absorbed, the solution was filtered and concentrated. Thirty-six grams of the residue was dissolved in 180 ml. of 6 *N* hydrochloric acid and heated to 70°. To this stirred solution 107 Gm. of iodine monochloride in 200 ml. of 6 *N* hydrochloric acid was added in twenty minutes while the temperature of the reaction mixture was kept between 65 and 70°. Over an eighty-minute period 800 ml. of water was added. The resulting suspension was cooled and filtered. The solid was purified through the sodium salt as described above. Crystallization from methanol afforded a crop of crystals, m. p. 178–180° (uncorr.). Wt. 36 Gm. A second crop was obtained from the filtrate. Wt. 13.0 Gm., m. p. 174–176° (uncorr.). After one more crystallization the triiodo acid melted at 180–181° (corr.).

[1] The analyses were carried out under the supervision of M. E. Auerbach and K. D. Fleischer of this Laboratory.

Anal.—Calcd. for $C_{10}H_{12}I_2NO_2$: C, 21.56; H, 1.81; I, 68.36. Found: C, 21.86; H, 1.68; I, 68.30.

α-*n*-Propyl-*m*-nitrocinnamic Acid (IV).—A mixture of 77 Gm. of *m*-nitrobenzaldehyde, 190.7 Gm of valeric anhydride, and 34.2 Gm. of triethylamine was stirred at 100–105° for twenty hours. The dark reaction mixture was treated with enough hydrochloric acid to neutralize the base and it was then steam-distilled. The nonvolatile oil set to a semisolid on cooling. It was filtered and pressed as dry as possible. The filter-cake was dissolved in dilute sodium hydroxide and the solution was washed with benzene. The extracts were discarded. The acid was precipitated with acetic acid. The brown solid was filtered and washed with water. The filtrate was acidified with hydrochloric acid and cooled.

The brown solid was recrystallized from benzene-ligroin and then dilute ethanol. It weighed 64.5 Gm. and melted at 122–125°. One more recrystallization raised the melting point to 125–127°.

Anal.—Calcd. for $C_{12}H_{13}NO_4$: N, 5.96. Found: N, 5.71.

The filtrate that was strongly acidified deposited 7.0 Gm. of crystals, m. p. 96–103°. After three crystallizations the solid melted at 109 to 111.5°. It is presumably the *cis* isomer. On admixture with the above isomer the melting point fell to 91–100°.

Anal.—Calcd. for $C_{12}H_{13}NO_4$: N, 5.96. Found: N, 6.07.

α-*n*-Propyl-β-(3-amino-2,4,6-triiodophenyl)-propionic Acid (X).—Sixty grams of the nitro acid was reduced in dilute sodium hydroxide solution with the aid of Raney nickel catalyst. After two hours, hydrogen absorption had ceased. The solution was filtered and treated with 600 ml. of glacial acetic acid.

This solution was stirred and warmed to 70°. In one hour 137 Gm. of iodine monochloride in 200 ml. of acetic acid was added. The mixture was stirred for six hours, cooled, and filtered. The solid was washed with 50% acetic acid and dried. Wt. 74.5 Gm., m. p. 152–155°.

The original filtrate was heated for four hours at 70° to furnish 8.0 Gm. of material, m. p. 159–162° after recrystallization from methanol.

The first crop of crystals was dissolved in 70 ml. of 2 *N* sodium hydroxide, diluted with 40 ml. of water and treated with 100 ml. of saturated salt solution. On cooling the sodium salt separated. The salt was collected on a filter and washed with salt solution and then acetone. The sodium salt was converted to the free acid and recrystallized from methanol twice followed by chloroform-petroleum ether. The yield of triiodo acid, m. p. 169.2 to 170.5° (corr.), was 19.7 Gm.

Anal.—Calcd. for $C_{13}H_{14}I_3NO_2$: I, 65.09; neutral equiv., 585. Found: I, 65.25; neutral equiv. 584.

In another experiment, run slightly differently both di- and triiodo acids were isolated.

One-tenth of a mole of the nitrocinnamic acid was reduced as above and the filtered aqueous solution of the sodium salt was made acid with strong hydrochloric acid. To this solution iodine monochloride (16.8 ml.) in 50 ml. of 6 *N* hydrochloric acid was added. After three hours at 70° the dark solid that separated was washed with water and dried. It was recrystallized from dilute methanol to furnish three crops of crystals. The first, m. p. 130–136°, weighed 26 Gm. The second amounted to 5.0 Gm. and melted at 140–144°. The last crop (3.0 Gm.) melted at 153–155°.

The third crop was recrystallized from benzene-ligroin and further purified through the sodium salt. After conversion of the latter to the free acid and recrystallization from methanol the compound melted at 159–160°. It was the diiodo acid.

Anal.—Calcd. for $C_{12}H_{13}I_2NO_2$: I, 55.29. Found: I, 55.85.

After two crystallizations from methanol the first crop gave a substance with m. p. 162.5 to 165°. The analysis indicated that it was somewhat impure triiodo acid.

Anal.—Calcd. for $C_{12}H_{14}I_2NO_2$: I, 65.09. Found: I, 62.39.

α-*n*-Butyl-*m*-nitrocinnamic Acid.—A mixture of 73 Gm. of caproic anhydride, 25.8 Gm. of triethylamine, and 25.8 Gm. of *m*-nitrobenzaldehyde was heated at 100° for twenty hours, acidified with 100 ml. of 6 *N* hydrochloric acid, and steam-distilled. The mixture was worked up as in the case of IV. The fraction precipitated with acetic acid weighed 21.3 Gm. after recrystallization from dilute ethanol. The analytical sample was recrystallized twice more from ethanol. M. p. 109.9 to 110.6° (corr.).

Anal.—Calcd. for $C_{13}H_{15}NO_4$: N, 5.62. Found: N, 5.90.

The solid obtained after acidification of the filtrate with hydrochloric acid was obtained in small yield. After several recrystallizations it melted at 95–98°. It was not further investigated.

α-*n*-Butyl-β-(3-amino-2,4,6-triiodophenyl)-propionic Acid (XI).—Eleven grams of the acid, V, was reduced in ethanol with the aid of Raney nickel catalyst. The filtrate from the reduction was taken to dryness and the residue then was dissolved in 6 *N* hydrochloric acid. A solution of 24 Gm. of iodine monochloride in 50 ml. of 6 *N* hydrochloric acid was added and after one hour at 70° the mixture was treated with 100 ml. of water. The solid that separated was filtered and suspended in 200 ml. of water at 70°. A solution of 10 Gm. of iodine monochloride in 25 ml. of hydrochloric acid was added. After four hours the dark solid was filtered and washed with water. The air-dried solid weighed 20 Gm., m. p. 150–160°. After two recrystallizations from methanol with the aid of Darco the product melted at 175.0 to 176.4° (corr.).

Anal.—Calcd. for $C_{13}H_{16}I_3NO_2$: C, 26.06; H, 2.69; I, 63.56. Found: C, 26.38; H, 2.58; I, 63.50.

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A Fluorometric Method for the Determination of Hydrastine in Hydrastis*

By EINAR BROCHMANN-HANSSEN and JOSEPH A. EVERS

A fluorometric method for the quantitative determination of hydrastine is described. This method, based on the oxidation of hydrastine to hydrastinine, has been found to be specific and has the advantage of being sensitive, accurate, and rapid.

Of the three alkaloids known to be present in *Hydrastis canadensis*, hydrastine is of greatest importance in therapy, although both berberine and canadine have some physiological activity (1). Hydrastine and canadine are very weak bases [K_b for hydrastine = 1.7×10^{-6} (2)] and can be extracted with ether in the presence of ammonia. Berberine is a strong base [K_b = 3.35×10^{-3} (2)] and is not liberated from its salts by ammonia but by alkali hydroxide. This difference in basicity of the alkaloids enables one to separate readily the total alkaloids as two fractions, one containing the hydrastine and canadine, the other containing the berberine (3, 4). As yet, no quantitative method has been described for the separation of hydrastine and canadine, and the amount of canadine normally present in hydrastis is not known. Chemically, canadine is *l*-dihydroberberine. It forms white crystals when pure, but is easily oxidized to berberine in the presence of light and air.

The official assay procedure for hydrastis (5) is, with minor modifications, the same as the method described in 1894 by Keller (6). The assay has a number of undesirable features: (*a*) It is a gravimetric procedure, and as such it includes small amounts of nonalkaloidal substances not easily removed during the purification process; (*b*) the assay is expensive, both with regard to materials used and time involved; and (*c*), it does not actually determine hydrastine but the sum total of hydrastine and canadine. This latter argument becomes important because we have no assurance that the ratio between hydrastine and canadine is constant.

The volumetric and colorimetric methods (7, 8) proposed for the assay of hydrastis may have certain advantages over the gravimetric procedure, but so far no method is available that is specific for hydrastine. Such a procedure would seem to be highly desirable, and the present work was directed toward this end.

EXPERIMENTAL

Upon oxidation, hydrastine gives a mixture of hydrastinine and opianic acid. This reaction is commonly used to identify hydrastine, since hydrastinine is easily recognized by its intense fluorescence. We have adapted this oxidative procedure to a quantitative method for the determination of hydrastine.

Reagents and Equipment

Pure hydrastine[1]
Quinine sulfate, U. S. P.
Sulfuric acid, approximately 0.1 *N*
Nitric acid, specific gravity 1.30
Ammonia T. S., U. S. P.
Ethyl ether, peroxide-free
Photofluorometer
Constant temperature water bath
100-ml. volumetric flasks

A spectroscopic examination of the fluorescent light emitted by hydrastinine obtained by oxidation of hydrastine as described below, showed no sharply defined line, but a continuous spectrum between the green and the blue lines of mercury with the apparent maximum intensity in the green.

The fluorescence was measured with a Coleman Photofluorometer (Model 12A) equipped with a B-1 primary filter and a PC-2 secondary filter. A B-1, PC-1 filter combination gives a higher reading, but has the disadvantage of giving a very high blank value. The sensitivity of the instrument was adjusted to a reading of 50 with a solution of quinine sulfate in 0.1 *N* sulfuric acid (3.6×10^{-4} mg./ml.).

Procedure.—Fifty milligrams of pure hydrastine was dissolved in 0.1 *N* sulfuric acid and the volume adjusted to 250 ml. Standard solutions containing from 0 to 6 mg./100 ml. were prepared by diluting the stock solution with 0.1 *N* sulfuric acid. Five milliliters of hydrastine solution and 10 ml. of nitric acid,[2] sp. gr. 1.30, were mixed in a 100-ml. volumetric flask and placed in a constant temperature water bath at 50° for thirty minutes. After cooling under running water, the flask was filled up to the mark with distilled water at 20°. Ten milliliters of this solution was diluted in another flask to 100 ml. with distilled water, and the intensity of the fluorescence was measured.

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Presented to the Scientific Section, A. Ph. A., Buffalo meeting, August, 1951.

[1] Hydrastine used in these experiments was obtained from Merck and Co. and recrystallized several times from ethanol, m. p. (corr.) 134°.

[2] It was found convenient to use an automatic pipette with a 1,000-ml. reservoir and a 10-ml. measuring chamber to deliver 10-ml. portions of nitric acid.

Various oxidizing agents were tried, including potassium permanganate, ceric sulfate, hydrogen peroxide, and nitric acid. The oxidation with nitric acid appeared to be the most easily controlled.

Figure 1 shows the influence of time and temperature on the development and decay of the fluorescence, using nitric acid as the oxidizing agent.

Hydrastinine is slowly decomposed when exposed to ultraviolet light. Therefore, only the first few readings should be used. The fluorescence is dependent on the temperature. In the present work, all readings were taken at 25°.

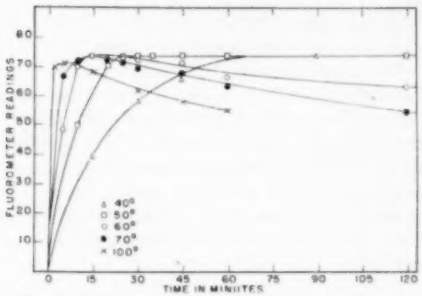

Fig. 1.—Development and decay of fluorescence by oxidation of hydrastine with nitric acid at various temperatures.

Figure 2 represents the standard curve for hydrastine.

The precision and accuracy of the method was investigated by assaying various known amounts of hydrastine. The results are listed in Tables I and II. In Table II hydrastine is first separated from berberine by extraction with ether in the presence of ammonia.

Adaptation of the Fluorometric Method to the Crude Drug.—In a 100-ml. Erlenmeyer flask were placed 0.400 Gm. of finely powdered hydrastis and 50 ml. of peroxide-free ether. The flask was stoppered with a cork of good quality and shaken for two minutes. One milliliter of ammonia T. S. was then added, and the mixture was shaken mechanically for thirty minutes. At the end of this time, 25 ml. of the ether extract was transferred to a 100-ml. volumetric flask, and the ether was removed almost completely on a steam bath. Ten milliliters of fresh ether was added and evaporated to approximately 2 ml. Finally 10–15 ml. of 0.1 *N* sulfuric acid was added and the ether removed completely. After cooling, the volume was adjusted to 100 ml. with 0.1 *N* sulfuric acid. Five milliliters of this solution was oxidized with 10 ml. of nitric acid in the manner described above. From the standard curve the content of hydrastine can be read directly in per cent ($X \times 10^{-4}$ mg./ml. corresponds to X per cent).

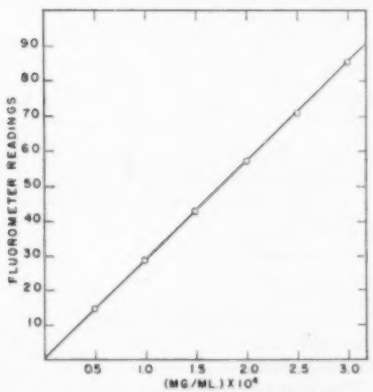

Fig. 2.—Standard curve for hydrastine.

TABLE I.—FLUOROMETRIC DETERMINATION OF PURE HYDRASTINE

Calculated, Mg.	Found, Mg.		Error, %
3.96	3.90, 3.92	3.91	−1.3
5.88	5.88, 5.88	5.88	0
5.00	5.02, 5.10	5.06	+1.2
23.30	23.25, 23.40	23.33	+0.1
12.80	12.65, 12.80	12.73	−0.5

TABLE II.—FLUOROMETRIC DETERMINATION OF THE HYDRASTINE CONTENT OF SOLUTIONS CONTAINING HYDRASTINE AND BERBERINE

Calculated, Mg.	Found, Mg.		Error, %
4.00	4.04, 3.98	3.99	−1.2
5.88	5.88, 5.85	5.87	−0.2
5.88	5.81, 5.83	5.82	−1.0

This fluorometric method was compared with the official assay procedure and also with the colorimetric method of Mendez, *et al.* (8). The results are given in Table III.

TABLE III.—A COMPARISON OF THE PROPOSED METHOD WITH THE N. F. METHOD AND THE COLORIMETRIC METHOD OF MENDEZ, *et al.*

	N. F. Method, %	Colorimetric Method, %	Fluorometric Method, %
	2.65	2.50	2.03
	2.61	2.54	2.06
	2.58	....	2.06
	2.63	....	2.04
	....	....	2.07
Av.	2.62	2.52	2.05

The short extraction time of the proposed method is sufficient for complete extraction of hydrastine, because no increase in concentration was found when the extraction was carried out according to the N. F.

Although the original acidic solution of impure hydrastine obtained from the ether extract is yellow

in color, the final dilution after oxidation is colorless to the naked eye. In order to determine whether or not the small amounts of impurities present in the final dilution might interfere with the production and measurement of the fluorescence by absorption of light, the absorption was determined in a Beckman Spectrophotometer (Model DU). A solution obtained from the same amount of pure hydrastine was used as a blank. Readings were taken at 10 mμ intervals from 250 to 500 mμ. The result was 100 ± 1% transmission. As a second check, the following experiment was performed: a solution of hydrastine (5 mg./100 ml.) was contaminated with enough berberine to produce a color comparable with the color of the impure acid hydrastine solution obtained from the crude ether extract. Upon oxidation and dilution, this solution gave a reading equal to an equivalent amount of pure hydrastine treated in the same manner.

dl-Canadine was prepared by reduction of berberine according to Neugebauer and Brunner (9). Five milliliters of a solution containing 5 mg./100 ml. was oxidized with nitric acid in the usual way, and the fluorescence measured after dilution to 2.5×10^{-4} mg./ml. Since canadine is oxidized to berberine, the same experiment was performed with this alkaloid. The results are given in Table IV.

TABLE IV.—FLUOROMETER READINGS OBTAINED BY OXIDATION OF THE SAME AMOUNTS OF *dl*-CANADINE, BERBERINE, AND HYDRASTINE

	Fluorometer Readings
dl-Canadine, 2.5×10^{-4} mg./ml.	0.5
Berberine, 2.5×10^{-4} mg./ml.	0.5
Hydrastine, 2.5×10^{-4} mg./ml.	71.
Blank	0.5

CONCLUSIONS

From the experiments just described, it may be concluded that the proposed method is specific for hydrastine and, therefore, offers definite improvements over other methods reported for its determination. In connection with another assay procedure which determines the sum of hydrastine and canadine, it is possible to determine the content of canadine in hydrastis by difference.

Because of the small amount of drug required and the better ratio of drug to extracting solvent, the time of extraction can be reduced considerably without sacrificing accuracy. Any further purification of the alkaloid appears to be unnecessary. This permits a considerable saving with respect to drug, reagents, and time. Once a standard curve has been constructed, a complete determination, including the extraction, can be performed within one hour and a half. The precision of the method is determined by sensitivity and reproducibility of the fluorometer employed.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Benzoquinonecarboxylic acid
Saccharic acid
Sodium oxaloacetate
D-Tagatose
n-Tetradecyldimethylamine
Tetrahydro- β-naphthylamine
Tetrazolium chloride
Thiobenzoic acid
Thyronine
γ-Trimethylbutyrobetaine
Leucylglycine
D-Mannuronic acid
4,6-Dihydroxyquinoline
Diphenylpyraline
L-Gulonic acid
3-Carboxy-9,10-dihydroxyanthracene
Copper abietinate
Cyclohexandiol-1,2
1,2-Dianhydromannitol
N-Acetylcystine

The Composition of Sapote Gum*

By ERNEST ANDERSON† and HARVEY D. LEDBETTER‡

Sapote gum is a mixture of 90 to 94 per cent water-soluble polyuronide and 6 to 10 per cent water-insoluble resin. The two substances can be separated by dissolving the polyuronide in hot water and filtering off the resin. The polyuronide consists of the calcium and magnesium salts of a methoxyuronic acid to which pentosan material is attached. There is approximately 1 methoxyl group to 2 uronic acid groups and 7 anhydro pentose groups in the complex acid. D-Xylose and L-arabinose are the only sugars present. Approximately 8.5 xylose groups are present for each arabinose group. A mild acid hydrolysis liberates all of the L-arabinose. Under these conditions most of the D-xylose remains attached to the uronic acid. More vigorous acid hydrolysis liberates most of the D-xylose. An aldobionic acid consisting of the methoxyuronic acid attached to one molecule of D-xylose remains.

SAPOTE GUM is available in Peru where it is used for sizing cloth, as a glue, and for the preparation of water paints. In this article the purification and determination of the composition of the gum are described. Over 90 per cent of it is soluble in hot water. It appears to be suitable for some industrial and pharmaceutical uses.

EXPERIMENTAL

Source.[1]—In Peru the gum is collected from species of sapote trees that grow in a semitropical climate near the equator at an elevation of 3,000 feet. When the trees are tapped, the latex, from which chicle is prepared, flows for a time. As this flow ceases, the gum slowly forms over the wound. Some samples received were dark colored and contained large amounts of impurities; others were light colored and contained very little impurity.

Purification.—The crude gum consists of two substances: (*a*) a water-soluble polyuronide varying in amount from 90 to 94%; (*b*) a water-insoluble resin varying in amount from 6 to 10%. The former is the true gum. The latter seems to be dried latex which became mixed with the polyuronide during gum formation. Only the polyuronide portion was investigated. It was separated from the resin by heating the crude gum with 10 times its weight of water in a bath of boiling water and filtering the cloudy solution first through cloth and then through paper. After repetition of this process the cold solution was decolorized by the addition of a small amount of liquid bromine. It was then poured into 5 volumes of ethanol. The granular precipitate was filtered at the pump and washed with ethanol and ether. After a second purification a white granular product resulted. The amounts of resin found in the unpurified gum were, respectively, 5.99 and 9.54% in the light and dark colored samples.

Preparation of the Free Gum Acid.—Selected samples of the gum were dissolved in hot water and filtered through cloth. The cold solution was mixed with excess of cold dilute hydrochloric acid. The free gum acid was precipitated from this solution by addition of 8 volumes of ethanol.

The Constituents.—The presence of a uronic acid in the gun was established by the naphthoresorcinol test, (1, 2), and by the evolution of carbon dioxide when it is heated in a 12% solution of hydrochloric acid (3). This acid has not yet been fully identified but indications of D-glucuronic acid were obtained. No indication of D-galacturonic acid was obtained. The presence of a methoxyl group, ether-linked to the uronic acid, was established by the methods of Denigès (4) and von Fellenberg (5). Pentosan determinations and tests for pentoses showed the presence of large amounts of these sugars (1, 6), and the absence of methyl pentoses. No trace of hexose sugars was observed at any time. A spectroscopic analysis of the ash showed the presence of large amounts of calcium and magnesium and no more than traces of other metals. The gum consists of the calcium and magnesium salts of a methoxyuronic acid combined with pentosan material.

Analysis of the Gum.—The results of the analysis of the unpurified and purified gum and the gum acid are given in Table I. The methods used in the analysis are standard and fully described in the literature (1, 6, 7). The low total percentages in columns 1 2, and 4 of Table I are due to the presence of resin in these fractions. The resin contained no pentosan, uronic acid, or methoxyl. The material in column 3 contained very little resin. Only about half of the uronic acid units are combined with a methoxyl group. This is also true of the uronic acid groups in the barium salts analyzed in Table II. On the average there are approximately 3.5 pentose units for each uronic acid unit in the complex molecule.

Hydrolysis of the Gum.—For hydrolysis the gum was dissolved in water and the solution made 4% acid with sulfuric acid. This solution was heated for varying lengths of time and degrees of temperature. It was neutralized by the addition of barium carbonate and heated with decolorizing carbon and concentrated *in vacuo* to a thin syrup. The barium salts were precipitated by the addition of the syrup to ethanol. The salts were further purified by redissolving them in water and adding to ethanol. The alcohol solution of the sugars, free of barium

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[1] The gum investigated was supplied by Asher, Kates and Co. of Lima, Peru, together with information as to its occurrence, method of collection, and uses.

TABLE I.—ANALYSIS OF UNPURIFIED AND PURIFIED SAPOTE GUM AND GUM ACID[a]

	Col. 1 Unpurified Gum	Col. 2 Gum Purified Once	Col. 3 Gum Purified Twice	Col. 4 Gum Acid
Moisture, %	12.13	8.68	6.54	6.30
Ash, %	4.27	2.05	2.09	.06
Carbon dioxide, %	5.92	6.44	6.64	6.27
Uronic acid, %	25.84	28.11	28.98	27.51
Pentosan, %	64.7	66.5	68.8	67.9
Methoxyl, %	2.59	2.41	2.45	2.86
CH_3 calculated from methoxyl, %	1.17	1.09	1.10	1.30
Total, %	91.71	95.70	98.88	96.71
Equivalent weight	684.	653.	655.	679.
Pentose units per uronic acid	3.64	3.44	3.46	3.61
Methoxyl unit per uronic acid	0.622	0.531	0.523	0.65

[a] The results have been corrected for moisture and ash. The equivalent weights are calculated from the uronic acid.

salts, was concentrated *in vacuo* and the sugars identified (1, 6, 7).

Hydrolysis for Two Hours at 80°.—The resulting barium salts 1 were purified by two additional precipitations from ethanol. The results of the analysis are given in column 1 of Table II. These salts have 2.31 pentose groups for each uronic acid group. Slightly more than one pentose group was removed during the hydrolysis. By dissolving the sugar syrup in a little water and ethanol, crystalline L-arabinose was isolated. It was identified by its $[\alpha]_D^{25} = +100.8°$ and by conversion to the diphenyl hydrazone melting at 199° (1, 6). The presence of some D-xylose in the syrup was established by Bertrand's test (1). The sugar syrup was not fermented by bakers' yeast and gave no mucic acid when oxidized with 20% nitric acid. This establishes the absence of fermentable hexoses. The sugars liberated during this hydrolysis consisted chiefly of L-arabinose with some D-xylose.

Hydrolysis for Four Hours at 80°.—Two lots of gum were hydrolyzed under these conditions. The treatment of the resulting barium salts 2 and 3 differed in that 3 was fractionated more than 2, and the less soluble fraction was analyzed. The results are given in columns 2 and 3 of Table II. They show that an average of two pentose groups remain combined with one uronic acid. The concentrated sugar syrups gave crops of crystalline sugars with specific rotations varying from $[\alpha]_D = +18°$ to $[\alpha]_D = +100°$. Usually glacial acetic acid gave more crystalline sugar with lower specific rotation. By using ethanol as solvent, some pure crystalline L-arabinose was obtained. An examination of these different crystalline sugars showed that they were all mixtures of D-xylose and L-arabinose. None of the noncrystalline syrups nor of the crystalline sugars were fermented by bakers' yeast or gave any mucic acid when oxidized with 20% nitric acid.

Hydrolysis of Barium Salts in the Autoclave.—Some of barium salts 1, 2, and 3 were dissolved in water and treated with sufficient sulfuric acid to precipitate all of the barium and leave 4% sulfuric acid in the solution. This solution was heated in an autoclave at 16 lb. gauge pressure (120°) for four hours. Barium salt 4 and a sugar syrup were isolated from the solution. The results of the analysis of this salt are given in column 4 of Table II. The data show that 0.72 of one pentose group remains combined with each uronic acid in the salt. Using glacial acetic acid as solvent, practically all of the sugar was obtained in crystalline form with $[\alpha]_D = +18.8°$. Only D-xylose was present in this syrup. No L-arabinose was present. All of the L-arabinose had been hydrolyzed off by heating the gum to 80° for two hours in a 4% solution of sulfuric acid. This suggests that the L-arabinose is present in the furanose form, attached to D-xylose.

Hydrolysis of the Gum in a Bath of Boiling Water.—The gum in a 4% sulfuric acid solution was heated for five hours in a bath of boiling water. The barium salt was isolated in the usual way. It was dissolved in water and treated with sufficient sulfuric acid to precipitate the barium and leave 4%

TABLE II.—ANALYSIS OF BARIUM SALTS FROM HYDROLYSIS OF SAPOTE GUM[a]

	Col. 1 Salts from Gum Heated for 2 Hr. at 80°	Cols. 2 and 3 Salts from Gum Heated for 4 Hr. at 80°		Col. 4 Salts from Barium Salts 1, 2, and 3 Heated in Autoclave for 4 Hr. at 120°	Col. 5 Salts from Gum Heated for 28 Hr. in Boiling Water Bath
Barium, %	13.14	14.43	14.20	22.72	23.43
Pentosan, %	55.5	50.5	52.6	25.9	26.1
Carbon dioxide, %	8.02	8.49	8.34	11.96	11.75
Uronic acid, %	35.02	37.08	36.42	52.23	51.54
Methoxyl, %	...	...	3.56	4.16	4.67
CH_3 calculated from methoxyl, %	...	...	1.60	1.88	2.11
Total, %	...	...	104.82	102.72	103.18
Equivalent weight	566.	531.	553.	377.	385.
Pentose units per uronic acid	2.31	1.98	2.1	0.72	0.74
Methoxyl units per uronic acid	...	...	0.61	0.494	0.564

[a] In all cases a 4% solution of sulfuric acid was used for hydrolysis.

sulfuric acid in solution. This solution was heated for twenty-three hours in a boiling water bath. Barium salt 5 was isolated in the usual way. The results of its analysis are given in col. 5 of Table II. These check closely the analysis of barium salt 4.

Discussion of the Results in Table II.—As previously explained, column 1 shows that when the gum is heated to 80° for two hours in a 4% solution of sulfuric acid all of the L-arabinose and a small amount of D-xylose are liberated. Columns 2 and 3 show that when the heating is for four hours under the above conditions two molecules of xylose still remain attached to the uronic acid. Columns 4 and 5 show that even very drastic hydrolysis in the autoclave or boiling water both fail to liberate all of the xylose. This difficulty in liberating the last sugar from a uronic acid that has a methoxyl group attached has occasionally been reported (7). The data in Table II show that the number of xylose groups that remain attached to the uronic acid depend on the conditions of hydrolysis.

Per Cent Araban in the Gum.—A weighed amount of the twice purified gum in a 4% solution of sulfuric acid was heated for three hours in a bath of boiling water. As a control 0.9742 Gm. of L-arabinose and and 1 Gm. of D-xylose were treated in the same way. The sulfuric acid was removed from these solutions and the barium salt was separated from the sugars. The L-arabinose in the two solutions was determined by the method of Neuberg (1). After correcting for the L-arabinose lost in the control, 7.2% araban was found present in the purified gum. Thus, for each L-arabinose group in the purified gum there are approximately 8.5 groups of D-xylose present.

SUMMARY

In Peru sapote gum slowly forms over the cuts made in sapote trees during collection of latex. The gum is a mixture of 90 to 94 per cent water-soluble polyuronide and 6 to 10 per cent of water-insoluble resin. The two substances can be separated by dissolving the polyuronide in hot water and filtering off the resin. The polyuronide consists of the calcium and magnesium salts of a methoxyuronic acid-pentosan complex. There is approximately 1 methoxyl group to 2 uronic acid groups and 7 pentose groups in the complex body. D-Xylose and L-arabinose are the only sugars present. Approximately 8.5 xylose groups are present for each arabinose group. Mild hydrolysis liberates all of the L-arabinose, which seems to be present in the furanose form. Most of the D-xylose remains attached to the uronic acid. Vigorous hydrolysis liberates most of the xylose, but leaves an aldobiuronic acid consisting of the methoxyuronic acid combined with D-xylose.

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Absorption and Excretion Studies of Certain Alkyl Sugar Derivatives*,†

By FLOYD R. SKELTON, HILDA M. McCONKEY, JOHN K. SOUCH, and GORDON A. GRANT‡

The absorption and excretion characteristics of several alkyl derivatives of glucose and fructose were studied in the rat and bladder fistula dog. When injected intraperitoneally in the rat these compounds were eliminated almost quantitatively in the urine. Following their oral administration the urinary excretion varied considerably. A diuresis accompanied the urinary elimination of relatively large amounts of these sugar derivatives. Of the sugar derivatives tested in the dog, 3-ethylglucose, 3-methylglucose, 1-methylfructose, and 3-methylfructose were the most efficacious in that order. Administered in repeated oral doses, 3-methylglucose was more effective in producing diuresis and loss of sodium than 3-ethylglucose. Administration of 3-methylglucose to rats as 20 per cent of their dietary intake for a period of one year resulted in no metabolic adaptation to the material and no gross or microscopic evidence of toxicity.

THE INTRODUCTION of a methoxyl group into various positions in the glucose molecule markedly alters the metabolism of this substance in the body. Csaky reported that glucose and 3-methylglucose were absorbed at equal rates from the intestine, while 2-methylglucose, 5-methylglucose and 6-methylglucose were absorbed more slowly (1). Recently Campbell (2), Campbell and Davson (3), and Young (4) have not only confirmed this observation on 3-methylglucose in the cat and rat, but have also shown that it was almost quantitatively excreted in the urine. As the behavior of only methyl derivatives of glucose had thus far been studied it appeared of interest to investigate some other alkyl derivatives of glucose and fructose for their biological characteristics as well as to extend the previous observations on 3-methylglucose.

MATERIALS AND METHODS

Rat

Adult, male albino rats, weighing 180–200 Gm. were employed in the initial absorption and excretion studies of alkyl sugar derivatives. After a twenty-four-hour fasting period, 500 mg of each of the following sugar derivatives, 3-methylglucose, 3-ethylglucose, 1-methylfructose, 3-methylfructose, 3-propylglucose, and 3-butylglucose, was administered in 50% aqueous solution to groups of six rats by oral gavage and intraperitoneal injection. Control groups were similarly treated with glucose. All rats were maintained in metabolism cages and allowed access to water *ad lib.* for the duration of the experiment. The urine was collected for forty-eight hours after treatment and the total reducing substances determined by the method of Harding and Grant (5). The difference between the values for the experimental and control groups was considered to represent the amount of alkyl sugar excreted in that period.

The absence of any appreciable diminution in the total reducing substances following incubation with washed suspensions of bakers' yeast, as described in the method, indicated the absence of "fermentable sugar," so that the reducing power of the urine was an index of the amount of 3-methylglucose excreted. This was confirmed by the amount of 3-methylglucose found when calculated from methoxyl values done on the diluted urine.

Balance studies were also done in which 3-methylglucose, 3-ethylglucose, 1-methylfructose, and 3-methylfructose were fed in the diet to groups of six young, male hooded rats. Similar control groups received a diet of the following composition: crude casein 18%, glucose 43%, cellu flour 25%, corn oil 8%, cod liver oil 2%, and salt mixture (Steenbock) 4%. The rats of each experimental group were fed the same diet with 20% of the cellu flour replaced by one of the above alkyl sugar derivatives. All diets were supplemented by adequate vitamins daily. A period of four days was allowed for the animals to become accustomed to the synthetic rations and the metabolism cages before beginning the experiment. The average food and alkyl sugar intake and urine volume of each group was measured daily throughout the experimental period. Both fecal and urinary total reducing substances were determined by the method of Harding and Grant (5), the differences between the values of the control and experimental groups being considered to represent the amount of alkyl sugars contained in the feces and urine respectively.

Since 3-methylglucose appeared to be the alkyl sugar derivative most readily absorbed from the intestine, the effects of its chronic administration were studied. Thus control groups of 15 male and female hooded rats were allowed *ad lib.* the basic diet outlined above, while homologous groups received the

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‡ Our thanks are due to Drs. W. L. Glen and G. S. Myers of our organic laboratories for the preparation of these sugar derivatives and also to Dr. G. Papineau-Couture for the spectrophotometric determinations of the urinary electrolytes.

same diet with 20% of the cellu flour replaced by 3-methylglucose. Monthly weight records were kept of all groups throughout the experimental period of one year. At the end of this time three rats from each group were placed in metabolism cages and similar balance studies to those previously described were performed in order to determine whether adaptation to the ingestion of 3-methylglucose had occurred. In addition, blood erythrocyte, leukocyte, and hemoglobin determinations were done on all rats. After the rats were sacrificed with chloroform, the organs were removed, weighed, fixed in Bouin's solution, sectioned, and stained with hematoxylin and eosin for microscopic examination.

Dog

Preliminary studies on the behavior of these alkyl sugar derivatives in the dog have also been done. A male dog weighing 20 Kg. was prepared with a permanent bladder cannula by the method of Huggins, *et al.* (6), thus allowing urine collection at any desired interval during the experiment. The same animal was used at weekly intervals in the study of the following sugars: 3-methylglucose, 3-ethylglucose, 3-methylfructose, and 1-methylfructose. After a twenty-four-hour fast, 15 Gm. of each sugar was administered orally in 50% aqueous solution followed by a 10-ml. distilled water rinse. Hourly urine collections were made for twenty-five hours starting one hour before the administration of the sugar. The volume of each urine sample was measured and its total reducing power determined by the method of Harding and Grant (5), while the sodium and potassium content was measured with the flame photometer. Two control experiments were run in which 40 ml. of distilled water alone and 15 Gm. of glucose were administered in place of the alkyl sugar.

The effect of repeated administration of 3-methylglucose and 3-ethylglucose was also studied. These sugars were given in six doses of 10 Gm. each in 50% aqueous solution at four-hour intervals with hourly urine collections for twenty-seven hours beginning one hour before the first dose of sugar. The same studies as those outlined above were done on these urine samples.

RESULTS

Rats

As shown in Fig. 1, 3-methylglucose was 75% excreted in the urine after oral administration and 70% thus eliminated after intraperitoneal injection. The other alkyl sugars were uniformly more quantitatively excreted than 3-methylglucose following their intraperitoneal injection, but showed considerable variation in urinary excretion, after oral administration. Thus, of the amount given, 25% of the 3-ethylglucose, 20% of the 3-methylfructose, 60% of the 1-methylfructose, 27% of the 3-propylglucose, and 35% of the 3-butylglucose, was recovered in the urine.

The results of the balance studies showed that rats fed a diet containing 20% 3-methylglucose ate less food initially than their controls fed the basal synthetic diet. However, the consumption of the 3-methylglucose diet increased so that the over-all

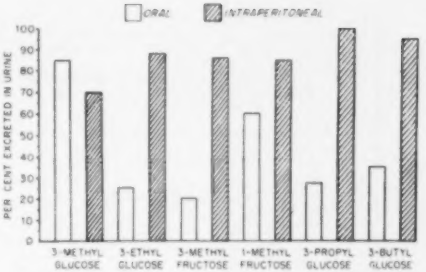
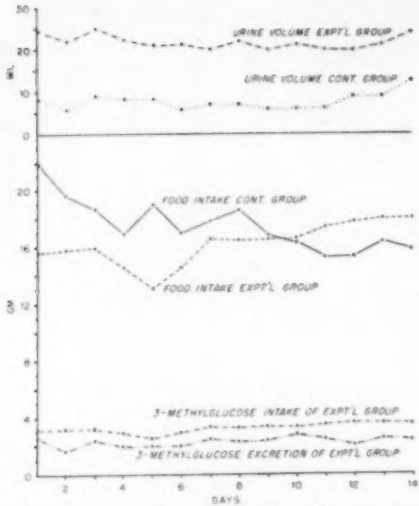


Fig. 1.—Urinary excretion of alkyl sugar derivatives in the rat following oral and intraperitoneal administration.

average daily food consumption of the control and experimental rats was 17.2 Gm. and 16.3 Gm., respectively (Fig. 2). The daily intake and urinary excretion of 3-methylglucose in the experimental group is also shown in Fig. 2. The average daily consumption of 3-methylglucose was found to be 3.3 Gm. per rat, while the urinary elimination averaged 2.3 Gm., as determined by the titration method of Harding and Grant, and 2.7 Gm. by methoxyl estimation. Thus from 70 to 76% of the ingested alkyl sugar was excreted in the urine under these conditions.

Since no significant difference was found in the total reducing power of the feces of control or experimental animals, all of the ingested 3-methylglucose was apparently absorbed. The daily urine volume of the experimental rats average 21.1 ml., a considerable increase over the 7.9 ml. excreted by the control animals (Fig. 2). In addition, 3-methylglucose did not appear to seriously disturb the normal growth of the animals, as the average final weights of the control and experimental rats were 225 Gm. and 211 Gm., respectively, both groups having averaged 165 Gm. at the beginning of the experiment.

In Fig. 3 it can be seen that the food intake of the rats receiving the other alkyl sugars was considerably less than their controls, hence necessitating the shorter experimental period. Thus the animals fed the diet containing 3-ethylglucose consumed only 11.6 Gm. daily while their controls averaged 21.1Gm. Those receiving 1-methylfructose and 3-methylfructose in their diet averaged only 9.9 Gm. and 7.5 Gm. per day, respectively, compared to their control average of 18.5 Gm. daily. In consequence the daily intake of these alkyl sugars was much less than in the 3-methylglucose experiment. The average intake was 2.3 Gm. for 3-ethylglucose, 2.0 Gm. for 1-methylfructose, and 1.5 Gm. for 3-methylfructose. In close agreement with the results of the experiments in which the sugar derivatives were given in a single oral gavage, the average urinary excretion of these compounds was also much lower than for 3-methylglucose. Thus an average of 0.8 Gm. of 3-ethylglucose, 1.07 Gm. of 1-methylfructose, and 0.31 Gm. of 3-methylfructose was excreted daily which represents 35, 53, and 19%, respectively, of the sugar intake. Consistent with the reduced sugar excretion was the lower daily urine volumes. The rats receiving the 3-ethylglucose diet averaged 12 ml. per



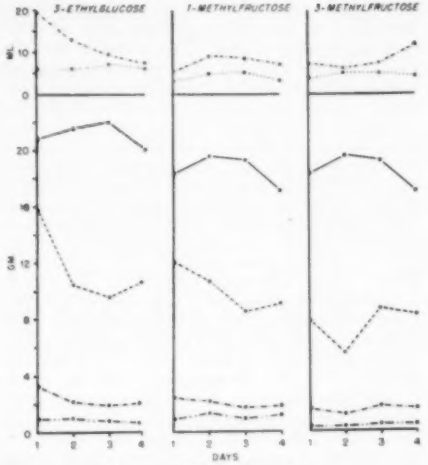


Fig. 2.—Results of balance study in which 3-methylglucose was fed to rats as 20% of the diet.
Fig. 3.—Results of balance studies in which 3-ethylglucose, 1-methylfructose, and 3-methylfructose were fed to rats as 20% of the diet.

day compared to 6 ml. for their controls. Similarly, the rats receiving the diets containing 1-methylfructose and 3-methylfructose excreted 7.5 and 8.0 ml. of urine, respectively, as compared to 4.5 ml. for their controls. The growth retardation of the rats fed 3-ethylglucose, 3-methylfructose, and 1-methylfructose was commensurate with their reduced food consumption during the short course of the experiments.

The results of the administration of 3-methylglucose to rats for a period of a year are presented in Table I. There was no growth retardation of either male or female rats receiving 3-methylglucose throughout this period. Furthermore, the balance studies performed after one year showed no adaptation to 3-methylglucose. A slight difference was noted between the values for male and female rats (Table I), but the average excretion of 65% was not significantly different from the 70% excretion observed in the previously described short-term experiment. The average twenty-four-hour urine volumes of approximately 7 ml. for the control and 20 ml. for the experimental animals were almost identical to those obtained in the short-term study. No differences were found in the red blood count, white blood count, or hemoglobin values between the control and experimental groups of either sex. The organ weights of the rats of either sex receiving 3-methylglucose did not significantly differ from those of the corresponding controls, and the gross and

TABLE I.—RESULTS OF TOXICITY STUDY IN WHICH MALE AND FEMALE RATS RECEIVED 3-METHYLGLUCOSE AS 20% OF THE DIET FOR A ONE-YEAR PERIOD[a]

	Controls		Experimental	
	Male	Female	Male	Female
Urinary per cent excretion 3-methylglucose	...	...	69	62
24-Hour urine volume, ml.	6.4	8.4	19	21
Blood erythrocytes	8,950,000	8,357,000	9,128,000	7,830,000
Blood leukocytes	16,300	10,000	14,660	14,000
Hemoglobin, mg./100 ml.	15.8	15.2	15.9	15.3
Liver	9654 ± 307	6184 ± 269	9788 ± 521	5988 ± 519
Kidneys	1827 ± 73	1329 ± 65	1933 ± 26	1291 ± 64
Heart	883 ± 13	660 ± 44	809 ± 21	612 ± 18
Brain	1360 ± 19	1249 ± 47	1360 ± 41	1177 ± 18
Pituitary	10.0 ± 0	10.3 ± 0.3	10.0 ± 0	10.0 ± 0
Adrenals	35.1 ± 1.4	43.6 ± 3.0	40.8 ± 2.5	37.8 ± 2.3
Spleen	1023 ± 63	718 ± 51	1160 ± 96	791 ± 44
Thyroid	17.9 ± 1.1	14.9 ± 1.3	15.8 ± 0.7	14.8 ± 0.3
Testes	2940 ± 165	...	2445 ± 183	...
Seminal vesicles	548 ± 43	...	658 ± 66	...
Prostate	255 ± 41	...	248 ± 21	...
Ovaries	...	27.2 ± 4.8	...	23.0 + 2.4
Uterus	...	312 ± 36	...	351 ± 66

[a] Organ weights are expressed in mg. accompanied by the standard error.

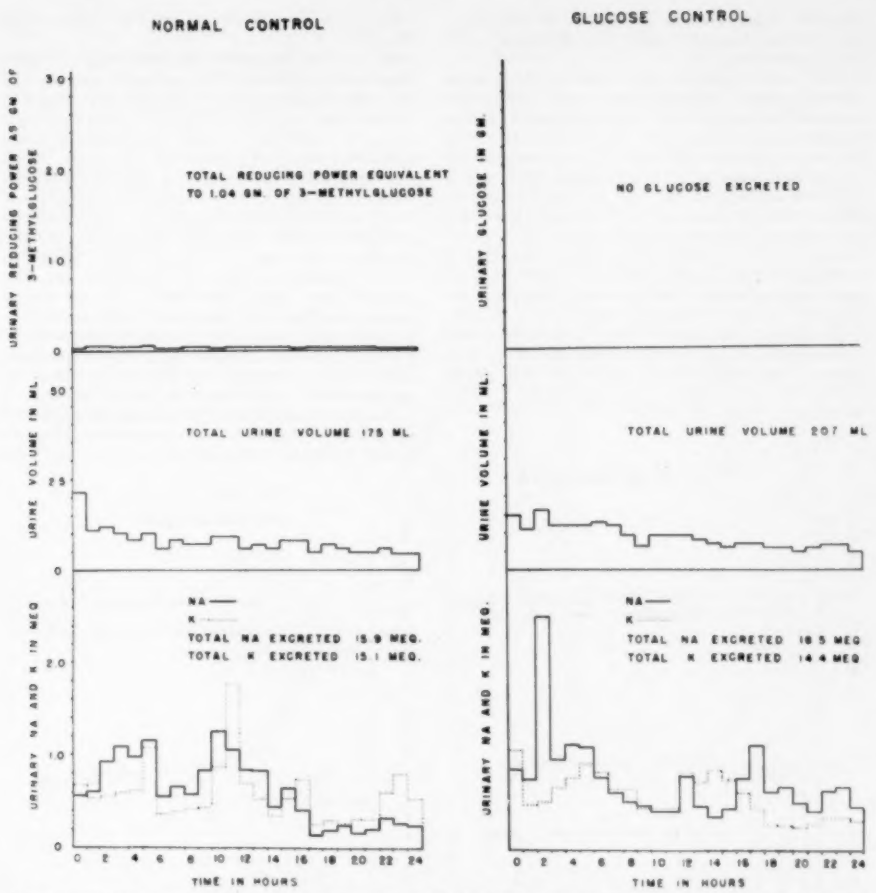

NORMAL CONTROL
GLUCOSE CONTROL
URINARY REDUCING POWER AS GM OF 3-METHYLGLUCOSE
TOTAL REDUCING POWER EQUIVALENT TO 1.04 GM. OF 3-METHYLGLUCOSE
URINARY GLUCOSE IN GM.
NO GLUCOSE EXCRETED
URINE VOLUME IN ML.
TOTAL URINE VOLUME 175 ML.
TOTAL URINE VOLUME 207 ML.
URINARY NA AND K IN MEQ.
NA
K
TOTAL NA EXCRETED 15.9 MEQ.
TOTAL K EXCRETED 15.1 MEQ.
TOTAL NA EXCRETED 18.5 MEQ.
TOTAL K EXCRETED 14.4 MEQ.
TIME IN HOURS

Fig. 4.—The hourly urine volumes and excretion of sodium (NA) potassium (K), total reducing substances and glucose for twenty-four hours in a normal and glucose-fed dog

microscopic examination likewise showed no apparent abnormalities.

Dog

Normal and Glucose Control Experiments.—The experimental results graphically presented in Fig. 4 are representative of several such control periods. The twenty-four-hour urine volume in the normal control experiment was 175 ml. and its total reducing power was equivalent to 1.04 Gm. of 3-methylglucose. This represents an average hourly excretion of approximately 40 mg. when calculated as 3-methylglucose. In all subsequent experiments with alkyl-sugar derivatives this value was recalculated in terms of the administered sugar and subtracted from the total reducing power of each hourly sample (also expressed as the administered sugar) to give the actual amount of sugar excreted. In the glucose control experiment the urine volume was 207 ml. with an excretion pattern identical to that of the normal control experiment. Since the total reducing power of the urine did not differ from that of the normal control period, it is evident that no glucose appeared in the urine.

The sodium and potassium excretion pattern was also essentially similar in both control experiments. Indeed the total sodium and potassium excretion in the twenty-four-hour normal control period was 15.9 and 15.1 milliequivalents (M. E.), respectively, while in the glucose control experiment they were 18.5 and 14.4 M. E.

Effects of a Single Administration of Alkyl Glucose and Fructose Derivatives.—Following the single administration of 15 Gm. of 3-methylglucose, 3-ethylglucose, 3-methylfructose, and 1-methylfructose, the 24 urine volumes were 303, 439, 239, and 258 ml., respectively, all of which were greater than the highest value observed for any control period. The maximum output occurred in the first three to four hours after 3-methylglucose (Fig. 5) and 1-methylfructose (Fig. 8), but lasted for eight to ten hours following the administration of 3-ethylglucose

(Fig. 6). Only a slight and transient increase in urine output occurred after 3-methylfructose administration (Fig. 7).

The urinary excretion of the alkyl sugars, sodium and potassium, paralleled the urine output, the maximum excretion occurring in the period of most marked diuresis (Figs. 5–8). The elimination of the sugars continued at a diminishing rate throughout the twenty-four hours after their administration so that 85% of 3-methylglucose, 75% of 1-methylfructose, 68% of 3-ethylglucose, and 17% of 3-methylfructose was excreted. However, some compensatory mechanism appears to cause an increased retention of sodium and potassium in the later hours since the urine content at that time decreased, in general, below the average value for the control periods. Consequently, the total loss of both sodium and potassium following a single administration of these sugars (Figs. 5–8) did not appear to differ significantly from the normal and glucose control values (Fig. 5).

Effects of Repeated Administration of Alkyl Glucose Derivatives.—The repeated administration of 3-methylglucose and 3-ethylglucose caused a periodic increase in urine, sugar, and electrolyte excretion at four-hour intervals corresponding to the administration of the sugars (Figs. 9 and 10). While the magnitude and pattern of the fluctuation in urine volume and sugar excretion remained relatively constant with both sugar derivatives, the electrolyte excretion showed less tendency to fluctuate, especially in the case of 3-ethylglucose (Figs. 9 and 10). Furthermore, despite the continued high urine secretion and sugar elimination, the amount of urinary sodium and potassium progressively decreased toward the end of the twenty-four-hour experimental period. Apparently a compensatory increase in the retention of these electrolytes has again occurred or the body has become depleted.

A total of 86.6% of the 3-methylglucose and 61.9% of the 3-ethylglucose was excreted during the period following their administration, while the

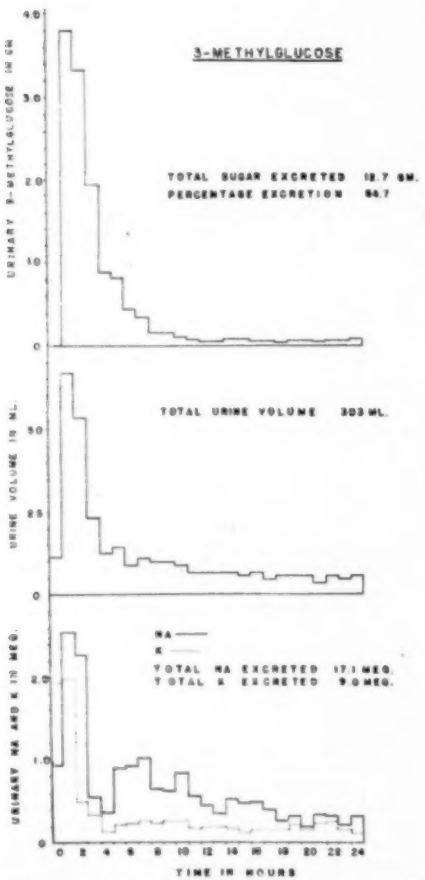


Fig. 5.—The hourly urine volumes and excretion of sodium (NA), potassium (K) and 3-methylglucose for twenty-four hours following the administration of the sugar to a bladder fistula dog.

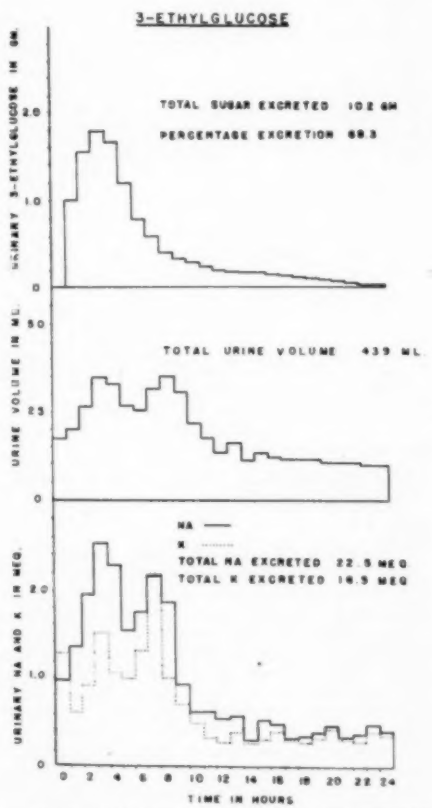


Fig. 6.—The hourly urine volumes and excretion of sodium (NA), potassium (K), and 3-ethylglucose for twenty-four hours following administration of the sugar to a bladder fistula dog.

urine volume totaled 858 ml. and 627 ml., respectively. Despite the decreased level of electrolyte [illegible]tion toward the end of the experiments, a total of 3[illegible]M. E. of sodium and 21.2 M. E. of potassium were lost with 3-methylglucose administration, and 32.5 M. E. and 14.8 M. E., respectively, were excreted during 3-ethylglucose administration. The sodium excretion was thus markedly increased after the feeding of both sugars, while the potassium excretion was only slightly elevated following repeated administrations of 3-methylglucose, and remained normal following similar 3-ethylglucose treatment.

DISCUSSION

These studies have confirmed in the rat the observations of other investigators (2–4) that 3-methylglucose is largely excreted in the urine following its oral or intraperitoneal administration. Other alkyl sugar derivatives investigated in this species have behaved qualitatively similar to but quantitatively different from 3-methylglucose. Thus all were nearly quantitatively excreted in the urine during the forty-eight hours following their intraperitoneal administration, but were excreted to a lesser extent than 3-methylglucose after oral gavage. Preliminary data on the absorption rates of 3-methylglucose and 3-ethylglucose in the rat suggest that the percentage excretion of each sugar derivative probably depends on the ratio between its rate of absorption and utilization by the organism.

When 3-methylglucose, 3-ethylglucose, 1-methylfructose, and 3-methylfructose were fed to rats as 20% of the diet, their urinary elimination was essentially the same as the percentage excretion following a single oral administration. Since relatively large amounts were excreted by the kidney, an osmotic type of diuresis resulted which was roughly proportional to the quantity of sugar contained in the urine. This was less apparent in the case of 1-methylfructose where the diuresis was relatively slight in proportion to the percentage excretion of the compound. The obvious distaste of the rats for

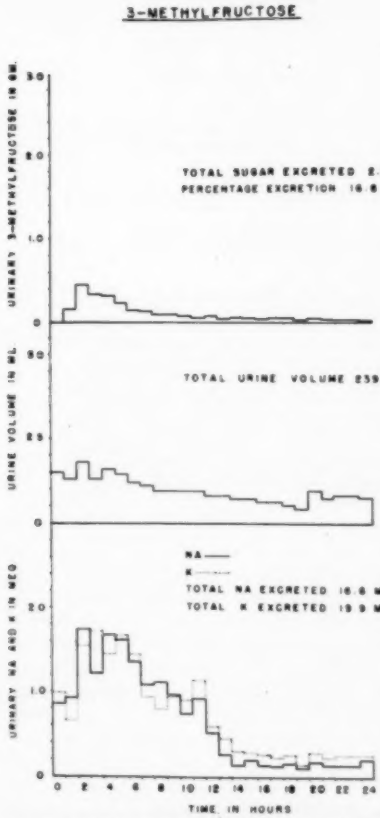


Fig. 7.—The hourly urine volumes and excretion of sodium (NA), potassium (K), and 3-methylfructose for twenty-four hours following administration of the sugar to a bladder fistula dog.

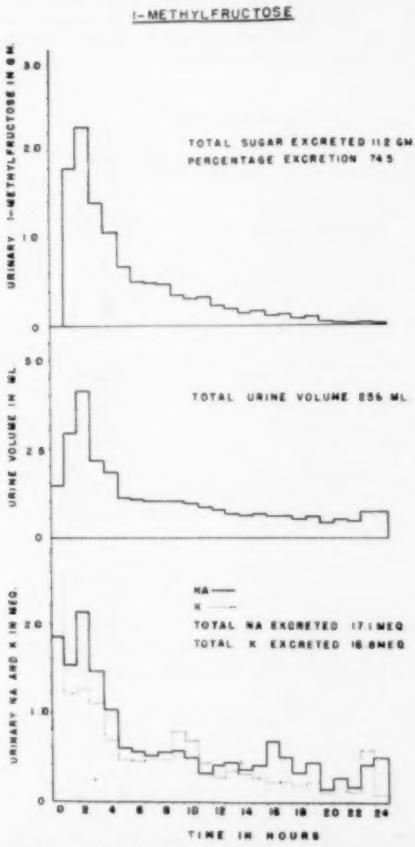


Fig. 8.—The hourly urine volumes and excretion of sodium (NA), potassium (K), and 1-methylfructose for twenty-four hours following administration of the sugar to a bladder fistula dog.

the diet containing either 1- or 3-methylfructose and their consequent reduced intake of water probably accounts for their relative ineffectiveness as diuretic agents.

The lack of any gross or microscopic evidence of toxicity following chronic administration of 3-methylglucose to both male and female rats for a period of one year as 20% of their dietary intake amply attests to the lack of toxicity of the material. Furthermore, during this time no apparent adaptation to the sugar derivative occurred since balance studies at the end of this period gave essentially similar results to those obtained in short-term feeding experiments.

In the preliminary study of these substances in a bladder fistula dog, the percentage excretion of 3-methylglucose and 3-methylfructose remained approximately the same as in rats, while the excretion of 3-ethylglucose was considerably greater. This probably accounts for the more pronounced diuretic effect of the latter sugar in this animal. In this dog, as well as in the rat, the percentage excretion of 1-methylfructose was greater than that of 3-ethylglucose, yet its diuretic action was only slightly better than 3-methylfructose, whose urinary excretion and diuretic properties were very low.

While the single oral administration of 3-ethylglucose produced a slightly more prolonged diuresis than 3-methylglucose, the latter compound appears distinctly preferable when repeated administrations are employed. The decreased efficacy of 3-ethylglucose under these conditions can undoubtedly be related to its slower intestinal absorption and the diarrhea and vomiting thereby produced. Thus, multiple oral administration seems essential for marked effectiveness of these substances, as diuretic agents. While these results have been obtained on a single dog only, the data appear sufficient to indicate the relative behavior characteristics of these sugar derivatives in this species.

SUMMARY

The biological properties of various alkyl derivatives of glucose and fructose have been studied in the rat and dog. When administered to the rat in a single intraperitoneal injection all alkyl sugar derivatives were nearly quantitatively excreted in the urine, 3-methylglucose, however, being excreted the least. Following oral administration

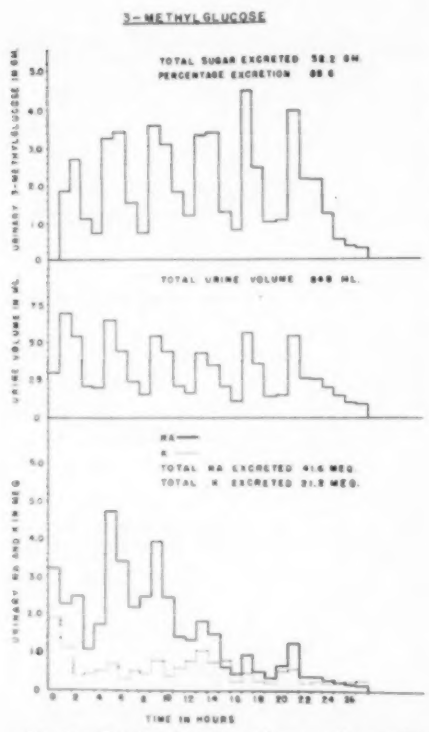


Fig. 9.—The hourly urine volumes and excretion of sodium (NA), potassium (K), and 3-methylglucose for twenty-seven hours, in a bladder fistula dog, the sugar being administered six times at four-hour intervals in the first twenty-four hours.

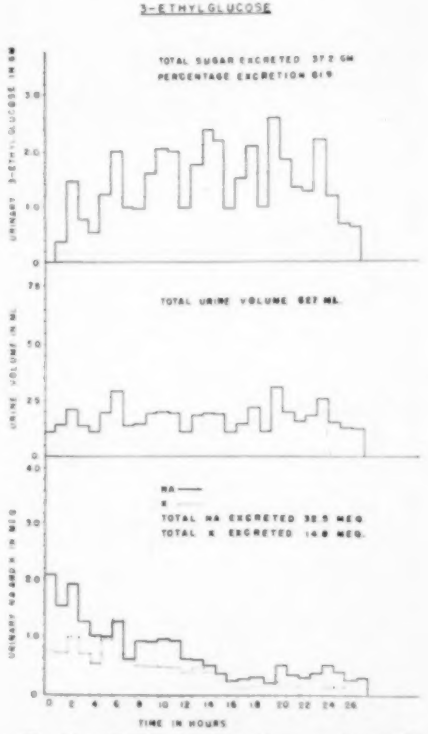


Fig. 10.—The hourly urine volumes and excretion of sodium (NA), potassium (K), and 3-ethylglucose for twenty-seven hours, in a bladder fistula dog, the sugar being administered six times at four-hour intervals in the first twenty-four hours.

by single gavage the percentage urinary excretion of these substances was as follows: 3-methylglucose, 85 per cent; 3-ethylglucose, 25 per cent; 3-methylfructose, 20 per cent; 1-methylfructose, 60 per cent; 3-propylglucose, 27 per cent; and 3-butylglucose, 35 per cent. When fed as 20 per cent of the diet the urinary elimination of the first four of the above substances remained essentially the same. Under these conditions increased amounts of water accompanied the excretion of the alkyl sugars, the diuresis being most pronounced for 3-methylglucose and 3-ethylglucose. Chronic feeding of 3-methylglucose as 20 per cent of the diet to male and female rats for a period of one year resulted in no gross or microscopic evidence of toxicity.

Preliminary study of these sugar derivatives in a bladder fistula dog showed the percentage urinary excretion following their single oral administration to be as follows: 3-methylglucose, 85 per cent; 3-ethylglucose, 68 per cent; 3-methylfructose, 17 per cent; and 1-methylfructose, 75 per cent. Diuresis and loss of sodium accompanied the excretion of these substances and was most marked with 3-methylglucose and 3-ethylglucose. In all cases the greatest urine output, sugar and electrolyte excretion occurred immediately after their administration. Following repeated doses of 3-methylglucose and 3-ethylglucose, the most efficient diuresis and loss of electrolyte was produced by the former compound.

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The Use of Some Imitation Flavors for Masking Distasteful Drugs. III. Castor Oil*, †

By DURWARD NEAL ENTREKIN‡ and CHARLES H. BECKER§

An investigation was made to determine the relative efficiencies of some imitation flavor concentrates, actually prepared from the basic ingredients, as masking agents for the initial and obnoxious tastes of castor oil. The imitation flavors were compared with some official flavoring agents. The concentrations of the flavors, sugar, and acid in the imitation flavored syrups were kept constant throughout the investigation. Further research should be carried out as to the effect of varying these concentrations. Results show that most imitation flavored syrups used in this investigation were significantly better than water for masking the disagreeable taste of castor oil. However, none of the flavors used, official as well as imitation, was significantly better than syrup, U. S. P.

In earlier papers (1, 2) studies were made to determine the relative efficiencies of some imitation flavors for disguising the taste of ammonium chloride and quinine hydrochloride. The flavor concentrates were actually produced from essential oils, aromatics, etc., that are employed in the flavor industry. This investigation is a continuation of the previous works; the same imitation flavors being used with castor oil as the distasteful drug. Some official flavors, including the aromatics of aromatic castor oil, N. F. IX, were used as the basis for comparison.

EXPERIMENTAL

The materials and formulas of imitation concentrates, preparation of syrups, taste test samples and controls, as well as the method of evaluation of the vehicles used in this investigation were the same as described in a previous paper (1). The aromatics of aromatic castor oil, N. F. IX, were incorporated in the external phase of the 50% castor oil emulsion and the taste test solutions prepared according to the standard procedure.

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† This paper is based on a thesis presented to the Graduate Council of the University of Florida by Durward Neal Entrekin in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
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The castor oil used was in the form of a 50% emulsion to facilitate its dispersion throughout the watery vehicle. The oil was of U. S. P. grade. Acacia was used as the emulsifying agent and the emulsion was produced in 6-oz. portions using a mortar and pestle.

Concentrations of Dispersions of Castor Oil Used.—Fourteen concentrations of the drug were used, 4 cc. of which constituted a taste test sample. The lowest number, No. 1, contained the smallest amount of oil and the concentrations increased in the ratio 1:2 with consecutively numbered samples. The numbers of the dispersions with the corresponding amount of emulsion per 180 cc. of prepared drink and the amount of drug contained in a 4-cc. portion of each concentration, which served as samples for the taste tests, are listed in Table I.

TABLE I.—CONCENTRATIONS OF CASTOR OIL USED

No. of Emulsion	Cc. of 50% Emulsion per 180 Cc. of Prepared Drink	Concentration of Drug in 4-Cc. Taste Test Sample, Cc.
1	0.009	0.0001
2	0.017	0.0002
3	0.034	0.0004
4	0.068	0.0008
5	0.136	0.0015
6	0.272	0.0030
7	0.544	0.0060
8	1.088	0.0121
9	2.176	0.0242
10	4.352	0.0484
11	8.704	0.0967
12	17.408	0.1934
13	34.816	0.3868
14	69.622	0.7746

TABLE II.—THE EFFECTIVENESS OF SOME IMITATION FLAVORED AND OFFICIAL SYRUPS ON THE DISGUISING POTENTIAL FOR CASTOR OIL

Syrup	No. of Subject	Mean Threshold in Water ± Standard Error, $m_2 \pm \sigma_2$	Mean Threshold in Syrup ± Standard Error $m_1 \pm \sigma_1$	Mean Disguising Potential ± Standard Error, $D \pm \epsilon$	Significance of Difference
Cacao syrup, U. S. P.	15	3.53 ± 1.30	9.60 ± 0.74	6.07 ± 1.50	4.05
Imitation coconut syrup	15	3.20 ± 1.42	9.06 ± 0.88	5.86 ± 1.67	3.51
Imitation wild cherry syrup	15	3.13 ± 1.30	8.93 ± 1.03	5.80 ± 1.66	3.49
Aromatics of aromatic castor oil, N. F.	15	3.50 ± 1.37	9.30 ± 1.18	5.80 ± 1.79	4.33
Aromatic eriodictyon syrup, N. F.	15	3.53 ± 1.30	9.00 ± 0.65	5.47 ± 1.46	3.75
Imitation raspberry syrup, acid added	16	4.06 ± 0.85	9.38 ± 1.20	5.32 ± 1.48	3.60
Imitation wild cherry syrup, acid added	16	4.06 ± 0.85	9.38 ± 1.03	5.31 ± 1.33	3.98
Imitation maple syrup	15	5.47 ± 1.13	10.33 ± 1.01	4.86 ± 1.51	3.21
Imitation grape syrup	15	3.13 ± 1.30	7.54 ± 0.92	4.41 ± 1.59	2.77
Imitation cream soda syrup	15	5.53 ± 1.60	9.54 ± 1.13	4.01 ± 1.95	2.05
Imitation butterscotch syrup	15	5.47 ± 1.13	9.47 ± 1.06	4.00 ± 1.55	2.59
Imitation raspberry syrup	15	5.53 ± 1.60	9.40 ± 1.35	3.87 ± 2.09	1.85
Imitation root beer syrup	15	3.20 ± 1.42	7.73 ± 1.11	3.53 ± 1.80	1.96
Imitation grape, acid added	16	4.06 ± 0.85	7.50 ± 1.21	3.44 ± 1.48	2.32
Glycyrrhiza syrup, U. S. P.	15	3.53 ± 1.30	8.73 ± 0.80	3.20 ± 1.52	2.11
Syrup, U. S. P.	15	3.13 ± 1.30	5.13 ± 0.92	2.00 ± 1.60	1.26

TABLE IV.—THE EFFECTIVENESS OF SOME IMITATION FLAVORED AND OFFICIAL SYRUPS ON THE OBNOXIOUS POTENTIALS FOR CASTOR OIL

Syrups	No. of Subjects	Mean Threshold in Water ± Standard Error, $m_2 \pm \sigma_2$	Mean Threshold in Syrup ± Standard Error, $m_1 \pm \sigma_1$	Mean Obnoxious Potential ± Standard Error, $D \pm \epsilon$	Significance of Difference
Aromatics of aromatic castor oil, N. F. IX	15	6.30 ± 1.57	12.30 ± 1.18	6.00 ± 1.96	4.28
Cacao syrup, U. S. P.	15	6.20 ± 1.42	11.87 ± 1.19	5.67 ± 1.85	3.06
Glycyrrhiza syrup, U. S. P.	15	6.20 ± 1.42	11.60 ± 1.18	5.40 ± 1.85	2.92
Aromatic eriodictyon syrup, N. F.	15	6.20 ± 1.42	11.40 ± 1.06	5.20 ± 1.77	2.94
Imitation coconut syrup	15	6.73 ± 1.03	11.70 ± 1.11	4.97 ± 1.51	3.28
Imitation wild cherry syrup	15	6.33 ± 1.68	11.00 ± 1.20	4.67 ± 2.06	2.27
Imitation maple syrup	15	7.67 ± 0.62	12.27 ± 1.10	4.60 ± 1.26	3.65
Imitation cream soda syrup	15	7.54 ± 0.74	11.87 ± 1.46	4.33 ± 1.64	2.65
Imitation raspberry syrup	15	7.54 ± 0.74	11.80 ± 1.32	4.26 ± 1.51	2.81
Imitation raspberry syrup, acid added	16	7.75 ± 0.58	11.94 ± 1.29	4.19 ± 1.41	2.97
Imitation wild cherry syrup, acid added	16	7.75 ± 0.58	11.89 ± 1.41	4.13 ± 1.52	2.71
Imitation butterscotch syrup	15	7.67 ± 0.62	11.73 ± 1.34	4.06 ± 1.47	2.76
Imitation root beer syrup	15	6.73 ± 1.03	10.20 ± 1.32	3.49 ± 1.67	2.08
Imitation grape syrup	15	6.33 ± 1.68	9.53 ± 0.99	3.23 ± 1.95	1.66
Imitation grape syrup, acid added	16	7.75 ± 0.58	10.44 ± 1.03	2.69 ± 1.18	2.28
Syrup, U. S. P.	15	6.33 ± 1.68	7.20 ± 1.38	0.87 ± 2.17	0.40

TABLE III.—THE SIGNIFICANCE OF DIFFERENCE BETWEEN THE MEAN DISGUISING POTENTIALS FOR CASTOR OIL WITH IMITATION FLAVORED AND OFFICIAL SYRUPS LISTED IN TABLE II

Cacao syrup, U. S. P.	Imitation coconut syrup	Imitation wild cherry syrup	Aromatics of aromatic castor oil, N. F.	Aromatic eriodictyon syrup, N. F.	Imitation raspberry syrup, acid added	Imitation wild cherry syrup, acid added	Imitation maple syrup	Imitation grape syrup	Imitation cream soda syrup	Imitation butterscotch syrup	Imitation raspberry syrup	Imitation root beer syrup	Imitation grape syrup, acid added	Glycyrrhiza syrup, U. S. P.	Syrup, U. S. P.
0.09				0.07	0.01	0.22									
0.12	0.03			0.08	0.22	0.43	0.20								
0.12	0.03	0.01		0.20	0.42	0.55	0.35	0.16	0.01						
0.29	0.18	0.15	0.14	0.49	0.54	0.64	0.40	0.18	0.05	0.05					
0.35	0.24	0.22	0.20	0.60	0.62	0.58	0.38	0.21	0.05	0.20	0.12				
0.38	0.26	0.23	0.22	0.68	0.57	0.80	0.56	0.37	0.18	0.26	0.17	0.04			
0.57	0.44	0.42	0.41	0.69	0.77	0.94	0.67	0.45	0.23	0.26	0.26	0.14	0.11		
0.76	0.63	0.61	0.58	0.76	0.90	1.05	0.78	0.55	0.33	0.37	0.26	0.64	0.66	0.55	
0.84	0.72	0.70	0.68	0.80	1.00	1.00	1.30	1.07	0.80	0.90	0.71				
0.96	0.82	0.79	0.76	0.89	1.02	1.08									
0.85	0.74	0.72	0.70	0.85	0.98	1.11									
1.08	0.95	0.93	0.89	0.96	1.06	1.15									
1.25	1.08	1.06	1.02	1.08	1.15	1.18									
1.34	1.18	1.15	1.11	1.61	1.53	1.60									
1.86	1.67	1.65	1.58												

TABLE V.—THE SIGNIFICANCE OF DIFFERENCES BETWEEN THE MEAN OBNOXIOUS POTENTIALS FOR CASTOR OIL WITH IMITATION FLAVORED AND OFFICIAL SYRUPS LISTED IN TABLE IV

Aromatics of aromatic castor oil, N. F.	Cacao syrup, U. S. P.	Glycyrrhiza syrup, U. S. P.	Aromatic eriodictyon syrup, N. F.	Imitation coconut syrup	Imitation wild cherry syrup	Imitation maple syrup	Imitation cream soda syrup	Imitation raspberry syrup	Imitation raspberry syrup, acid added	Imitation wild cherry syrup, acid added	Imitation butterscotch syrup	Imitation root beer syrup	Imitation grape syrup	Imitation grape syrup, acid added	Syrup, U. S. P.
0.13	0.10														
0.22	0.18	0.18		0.12	0.03										
0.30	0.29	0.29	0.10	0.19	0.13	0.13									
0.42	0.36	0.36	0.20	0.29	0.16	0.17	0.03	0.03							
0.47	0.48	0.48	0.27	0.33	0.19	0.22	0.06	0.06	0.03						
0.60	0.54	0.54	0.36	0.38	0.21	0.24	0.09	0.10	0.06	0.03					
0.66	0.59	0.59	0.40	0.39	0.24	0.28	0.12	0.34	0.32	0.28	0.26				
0.70	0.64	0.64	0.45	0.43	0.24	0.53	0.36	0.42	0.40	0.36	0.34	0.10			
0.75	0.64	0.64	0.46	0.45	0.27	0.45	0.43	0.82	0.82	0.76	0.73	0.39	0.24		
0.76	0.64	0.64	0.50	0.46	0.29	0.51	0.59	0.82	0.81	0.76	0.73	0.39	0.24		
0.80	0.68	0.68	0.50	0.43	0.29	0.53	0.36	0.42	0.40	0.36	0.34	0.10			
0.98	0.88	0.88	0.70	0.66	0.45	0.83	1.12	0.82	0.82	0.76	0.73	0.39	0.24		
1.00	0.91	0.91	0.75	0.71	0.51	0.59	0.81	1.28	1.28	1.23	1.22	0.96	0.81	1.14	
1.44	1.37	1.37	1.18	1.19	0.83	1.12	1.49	1.28	1.28	1.23	1.22	0.96	0.81	1.14	
1.78	1.68	1.68	1.54	1.55	1.27	1.49	1.28	1.28	1.28	1.23	1.22	0.96	0.81	1.14	

The results in Table II indicate that cacao syrup, U. S. P. XIV, is the best disguising agent for the initial taste of castor oil, followed closely by imitation coconut syrup, imitation wild cherry syrup, and the aromatics of aromatic castor oil, N. F. IX, in the order named.

All of the vehicles, with the exception of imitation raspberry syrup and syrup, U. S. P., were significantly better than water as disguising agents for the taste of castor oil. This is illustrated by a value of 1.96 or greater as the significance of difference. This value indicates that the difference is significant and not due to inherent variation in the subjects.

The addition of citric acid to the imitation flavored syrups—grape, raspberry, and wild cherry—did not cause a significant change in their disguising power.

The results in Table III indicate that none of the flavored vehicles used were significantly better than syrup, U. S. P., for disguising the taste of castor oil. This fact may be seen by noting that none of the syrups obtained a value of 1.96 or greater when compared to each other and to syrup, U. S. P.

The addition of citric acid to the imitation fruit-flavored vehicles caused no significant variations.

The results in Table IV show the aromatics of aromatic castor oil, N. F. IX, gave the highest potential for masking the obnoxious taste of castor oil. The official flavored syrups—cacao, U. S. P., glycyrrhiza, U. S. P., and aromatic eriodictyon, N. F.—compared favorably with the aromatics of the N. F. IX product.

Imitation coconut syrup had the highest mean obnoxious potential of the imitation flavored syrups. The addition of citric acid to the imitation fruit-flavored vehicles gave no significant advantage.

All syrups, with the exception of imitation grape and syrup, U. S. P., were significantly better than water as masking agents for the obnoxious taste of castor oil. This is indicated by a value of 1.96 or greater as the significance of difference.

The results in Table V show that none of the flavored vehicles used were better than syrup, U. S. P., in disguising the obnoxiousness of castor oil. The addition of citric acid did not enhance the results obtained with the fruit-flavored imitation vehicles.

DISCUSSION

Results show that most imitation flavored syrups used in this investigation were significantly better than water for disguising the disagreeable taste of castor oil. However, none of the syrups used, official as well as imitation flavored, were significantly better than syrup, U. S. P.

Cacao syrup, U. S. P., gave the highest mean disguising potential, while the aromatics of aromatic castor oil, N. F., gave the highest mean obnoxious potential of the syrups used. Imitation coconut syrup gave the highest disguising and obnoxious potentials of the imitation flavored syrups.

The abilities of the syrups to disguise the taste and the obnoxiousness of castor oil were not consistent. This fact indicates that a vehicle which would disguise the initial taste of the oil would not necessarily be of any great value in masking its obnoxiousness.

The addition of citric acid to the imitation fruit-flavored syrups did not appear to enhance the disguising ability of these syrups.

Flavor concentrations as well as the sugar and acid concentrations of the imitation flavored syrups were kept constant throughout the investigation. There were reports by some subjects that these concentrations were too high and others thought them too low. An investigation varying these factors should yield interesting results.

A majority of the subjects reported the official syrups—glycyrrhiza and aromatic eriodictyon—to be distasteful in themselves, but these syrups gave relatively high obnoxious potentials. This may be due to a synergistic effect where one obnoxious taste tends to cover up or reduce another.

The magnitude of the standard errors was consistently large throughout the investigation. Some factors which may have been responsible for these errors are as follows: castor oil does not have an acute taste; a majority of the subjects had a "phobia" of the taste of castor oil which would tend to produce inconsistent results; and none of the syrups used had a sufficient disguising ability for castor oil.

SUMMARY

Although there has been some work using imitation flavors to disguise the taste of distasteful drugs, there has been little or no work done on disguising the taste of castor oil with imitation flavors actually produced in the laboratory from chemicals, aromatics, etc., normally used in the preparation of these concentrates.

This investigation was made to determine the relative efficacy of some imitation flavored and official syrups as disguising agents using castor oil as the obnoxious-tasting drug.

It was found that most of the syrups used were significantly better than water as disguising agents for castor oil. None of the syrups used, however, were significantly better than syrup, U. S. P. Even the combination of flavors in aromatic castor oil, N. F. IX, is not significantly better than syrup, U. S. P.

Further work to disguise the disagreeable taste of castor oil is necessary. Different flavors, variation in concentration of flavor, sugar and other synergists, etc., should be considered for additional work.

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Note

A Note on the Influence of Sex upon the Response of the Rat Heart to Ouabain*, †

By ALBERT WOLLENBERGER and MAX L. KARSH‡

Holck and Kimura (1, 2) have shown that a marked sex difference in resistance to the toxic effect of ouabain is present in the mature rat. They found that whereas in immature animals the LD_{50} was alike in the two sexes, it was almost twice as high in four-month-old females as in males of the same age. In later work (3) a 53% difference was found in the four-month-old group. The present work was undertaken in order to determine whether the sex difference in the resistance of rats to ouabain is a property of the heart.

EXPERIMENTAL

Methods.—Langendorff preparations of the hearts of four- to five-month-old male and female rats of the Sprague-Dawley strain were used. The hearts were removed under ether anesthesia and attached to a perfusion apparatus for continuous reperfusion. The perfusion fluid consisted of 15 cc. of glucose-Locke-Ringer solution prepared according to the formula of Chenoweth and Koelle (4) and equilibrated with 95% O_2 and 5% CO_2. Its temperature upon reaching the heart was 39.0 ± 0.5°. After ejection from the heart the fluid was pumped by means of a roller-bearing pump into a reservoir in which it stood at a level of 56 cm. above the opening of the aortic cannula. The contraction of the heart was recorded on a kymograph by means of a wooden writing lever connected with a clip to the apex of the heart. After a period of ten to twenty minutes following attachment of the heart, during which amplitude of contraction and heart rate became constant, ouabain (Penick U. S. P., containing 20% water of crystallization) was administered at a rate of 0.25 mg. every three minutes until the heart became arrested in contracture. Increasing the rate as much as twofold did not significantly alter the lethal dose, which probably indicates that the maximal rate of uptake of the drug by the heart was achieved (5). After termination of the experiment the heart was dried to constant weight at 105°, and the lethal dose was calculated on the basis of dry heart weight.

RESULTS AND DISCUSSION

Two series of experiments were performed: one in May and June, and the other in November and December. As Table I shows, the lethal doses of ouabain in the November and December experiments were higher than in May and June, particularly in the males. In both series the hearts of the females required more of the glycoside than did the males. The sex difference was 45% in May and June and 31% in November and December and was in both instances statistically highly significant. It approaches in magnitude that reported by Holck and Kimura (3) for the four-month-old rat. Since death caused by ouabain is due to the lethal effect of the drug on the heart, our data show that the sex difference in the sensitivity of the mature rat to ouabain is largely accounted for by a differing sensitivity of the male and female heart.

Table I.—Minimum Lethal Dose of Ouabain for the Isolated Heart of the Four- to Five-Month-Old Male and Female Rat

Date of Experiment	Sex	No. of Animals	Mean Body Weight, Gm.	Mean Dry Heart Weight, Gm.	MLD per Gm. Dry Heart — Range, Mg.	MLD per Gm. Dry Heart — Mean ± S.E., Mg.
May and June	M	5	329	0.199	17.06–20.00	18.91 ± 0.61[a]
	F	5	240	0.145	25.46–30.55	27.73 ± 0.89[a]
November and December	M	7	311	0.176	23.73–37.29	29.18 ± 2.14[b]
	F	5	237	0.146	33.78–44.52	38.27 ± 1.98[b]

[a] Difference between the means significant ($P < 0.01$).
[b] Difference between the means significant ($P = 0.01$).

In no instance can be given at present for this different sensitivity. It has been reported (6, 7) that in the isolated frog heart both male and female sex hormones have an action resembling that of the cardiac glycosides. Whether administration of these hormones, to which the cardiac glycosides are structurally related, can modify the heart's response to the latter drugs, is not known. Holck and Kimura (1, 2) have reported that estrogens do not alter the resistance of the castrated female rat to ouabain, and they have found variable effects of castration on the resistance of both male and female rats, depending on the anesthetic used and on the mode of administration of the cardiac glyco-

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side. Exploratory experiments undertaken by us indicate that castration has no effect on the lethal dose of ouabain for the isolated heart of the female rat and produces only a slight increase in the lethal dose for the heart of the male rat.

SUMMARY

The lethal dose of ouabain for the isolated heart of the four- to five-month-old female rat is considerably higher than that for the heart of the male rat of the same age.

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Book Reviews

Biology for Pharmaceutical Students and Others. 2nd ed. By S. MANGHAM and A. R. HOCKLEY. Bailliére, Tindall and Cox, London, 1951. viii + 634 pp. Illustrated. 14.5 x 23.5 cm. Price $6.50.

This text is well adapted for biology courses in the pharmacy curriculum which combine the teaching of botany and zoology. The text has been designed primarily for pharmacy students of Great Britain who must prepare adequately for the Intermediate Examination (a type of basic science examination), and later for the Qualifying Examination of the Pharmaceutical Society. Great stress is placed upon physiological principles at the introductory level as these pertain to plant and animal life.

The text is planned in such a manner that the first 19 chapters deal pretty much with botanical aspects, whereas chapters 20 to 32 are concerned with animal biology. The final chapter, 33, outlines the principles of evolution, variation, and heredity as these are of concern among both plant and animal organisms.

Botanical treatment includes much introductory plant physiology such as factors of cellular absorption, respiration, enzyme activity, growth, and cell division. Chapters dealing with general plant macro-morphology are arranged beginning with stems and leaves, and followed by those on root, root systems, and seeds. Then elementary plant physiology is introduced and this is followed by several chapters which cover a combination of micro- and macro-morphology of plant parts with considerable emphasis upon function. The student is thereby attracted to some plant histology by a functional approach. This is very desirable and renders introductory plant anatomy more dynamic. Unfortunately space prohibits more than casual reference to these points.

It appears obvious that taxonomical characteristics of the four major plant divisions, thallophytes, bryophytes, pteridophytes, and spermatophytes, so often extensively covered in botany textbooks, are kept to a minimum in this textbook. Outstanding taxonomical characteristics of importance among subdivisions of these four divisions, such as are governed by reproduction and nutritional requirements, are nevertheless stressed. In this way the pharmacy student may learn *general* differences among large plant groups. Type examples used for illustration make use of traditional drug plant material and thus a twofold objective is achieved.

That portion of the text which deals with the animal body and its functions should be of great interest to the animal physiologists and pharmacologists of America who in recent years have clamored for more vertebrate zoology in biology or zoology undergraduate pharmacy courses. Two chapters are arranged early in the zoological treatment and these stress the comparative anatomy and function of the vertebrates. No attempt, however, has been made to cover all aspects of comparative vertebrate anatomy or physiology. Such chapters are followed by those on animal histology and embryology. The latter chapters do not deal as much with tissue histology as with tissue physiology and hence lend themselves very well with the trend to introduce students to animal physiology in zoology or biology courses. Nerve and muscle physiology as these are introduced in the text, while much abbreviated do acquaint the student early in his career with mammalian terminology and function. After all, it is this type of zoology with which pharmacy students are most concerned in follow-up courses in physiology, pharmacognosy, and pharmacology.

Traditional invertebrate zoology is not entirely omitted from this, despite a greater emphasis upon vertebrate zoology. In fact, separate chapters are devoted to the structure, physiology, and life histories of such lower forms as the amoeba and paramecium, hydra, the earthworm, insects, and representative body parasites from plasmodium species to liver flukes, and the tape worm. There has been no attempt made to arrange the zoological discussion according to a phyla sequence and unlike certain traditional college textbooks of zoology, the phylogenetic arrangement of animal organisms is entirely

lacking in this book. We believe the authors have arranged the zoological material wisely in this respect and that their system is more functional from the pharmacy student's standpoint. It appears fallacious under the circumstances of his basic science training program to further introduce the age-old arguments of which came first "the hen or the egg."

The text is well illustrated with original drawings and diagrams. Several laboratory demonstrations and experiments are suggested. It would appear, however, that an instructor would have need of a separate laboratory manual of his own choosing in order to satisfactorily stress his laboratory teaching. This could be easily outlined in conjunction with the Mangham-Hockley book.—H. W. YOUNGKEN, JR.

Hormones and Body Water. By ROBERT GAUNT and JAMES H. BIRNIE. Charles C Thomas, Publisher, Springfield, Ill., 1951. vii + 57 pp. 14.5 x 22.5 cm. Price $2.25.

This book is a concise compilation of the latest information on the relation of the endocrine system to the normal and pathological physiology of fluid metabolism. The kidney is described as keeper of the gate in the maintenance of a constant internal environment in the face of influences tending to vary the body content of salt and water, by adjusting the renal rate of excretion of these substances to maintain a constant osmotic pressure. A very interesting review of the possible evolution of the fluid, which accounts for 70% of the human body weight, and the factors that might have influenced its salt content is given.

Brief reviews lead to summaries of the action on the body fluid by: the posterior pituitary antidiuretic hormone; interrelation of adrenal cortical and posterior pituitary hormones; the thyroid; the adrenal medulla; the pancreas; the gonadal hormones; and the liver. Discussions on the toxemias of pregnancy, water intoxication, and water metabolism in the newborn are included.

The conclusions of this absorbing review start with the observation that water is the most voluminous body constituent and the most immediately essential item of the diet, and its metabolism is influenced by most factors which affect protoplasmic activity. The final observation is that despite much study, details of the complex mechanisms involved are incompletely known in many respects, but much progress in understanding has been made and more can be anticipated. An alphabetically arranged bibliography of 132 references is appended.

Organic Syntheses. Vol. 31. R. S. SCHREIBER, Editor-in-Chief. John Wiley & Sons, Inc., New York, 1951. vi + 122 pp. 15 x 23.5 cm. Price $2.75.

This volume of *Organic Syntheses* contains directions for the preparation and practical purification of 41 compounds. Many of the compounds are important as intermediates in the syntheses of pharmaceutically interesting products.

The established system of checking all the published procedures, and the inclusion of notes explaining particular steps in the syntheses are continued. The index includes material only from Volumes 30 and 31. For earlier material one must consult the indexes of Collective Volumes 1 and 2, and Volume 29.

Ethylene and Propylene Glycols. By JOHN RAE. The Pharmaceutical Press, London, 1951. 30 pp. 13.7 x 21.7 cm. Price 2*s.* 6*d.*

This pamphlet is a compilation from 12 articles on the solvent and preservative properties of ethylene and propylene glycols. It is not a comprehensive coverage of the applications of these glycols, but information of basic value is presented. The original articles appeared in the *Quarterly Journal of Pharmacy and Pharmacology* and in *The Pharmaceutical Journal*, both of which are published by the Pharmaceutical Society of Great Britain.

Selective Toxicity with Special Reference to Chemotherapy. By ADRIEN ALBERT. John Wiley & Sons, Inc., New York, 1951. xii + 228 pp. Illustrated. 11 x 17 cm. Price $1.75.

This book about selectively toxic agents which injure some kinds of cells and not others is more concerned with the physical and chemical means of action than with what the action accomplishes. The text is a compilation of lectures which are very well prepared and which are presented in an interesting and readable style. The author's interest in means rather than ends of reactions causes him to pass too quickly by certain developments. He states that flies and other biting pests are being controlled with derris and DDT. The development of DDT-resistant species is not mentioned.

The subject is projected in a manner to stimulate fundamental thinking and correlating of known facts to the end that underlying principles of selective toxicity, which has been a branch of technology rather than of science, can be discovered. The relation between the different fields of application is very well developed by presenting the agricultural applications, then leading into animal reactions. Different means of "accumulation" of toxic substances are illustrated in plants by the spraying of solutions over a field in which desirable smooth-bladed grains and undesirable coarse and retentive leafed weeds are growing. In this instance the toxic material "spared the wheat and spoiled the weed." Methods of accumulation and localized biological activity depending upon a specific type of reaction in certain cells of the animal body are discussed.

Dr. Albert pleads for a planned approach to the studies in the field of selective toxicity, and especially in the branch of chemotherapy. He stresses the fact that most of the efforts are now expended upon specific and limited studies to develop products for immediate application. The book is a very good review of the knowledge in this field and its size invites pocket carriage for reading at convenient times and places. An alphabetically arranged bibliography and subject index are appended.

Vitamin Methods. Vol. II. Edited by PAUL GYÖRGY. Academic Press, Inc., New York, 1951. xi + 740 pp. Illustrated. 16 x 23.5 cm. Price $14.50.

This book is a compilation of treatises prepared by specialists in the related divisions of biological, chemical, and physicochemical methods of vitamin study and testing. Dr. György, in this concluding volume, attempts to keep this work abreast of the forward pressure of research in this field, by includ-

ing a supplement to the chapters in Volume I on physical, chemical, and microbiological methods covering the work up to 1949–1950.

This volume presents the more specific methods used in vitamin research, such as animal assays and clinical appraisal of conditions based on the deficiency of vitamins. It is generally accepted throughout the whole development of vitamin research that compared with the physical, chemical, and the microbiological methods, animal assays reflect more truly the nutritional value of vitamins. The material is presented under the following headings: General Aspects of Small Animal Experimentation, by N. B. Guerrant; The Animal Vitamin Assays, by C. I. Bliss and Paul György; Laboratory Diagnosis of Human Vitamin Deficiencies, by James Hazlitt Jones; Clinical Signs of Malnutrition, by Norman Jolliffe; and Statistical Methods in Vitamin Research, by C. I. Bliss. The supplementary material is given in a 100-page section under the headings: Physical Methods of Vitamin Assay, by Eric T. Stiller; Chemical Methods of Vitamin Assay, by Paul György, Saul H. Rubin, and Elmer De Ritter; and Microbiological Methods in Vitamin Research, by Lemuel D. Wright, Helen R. Skeggs, S. H. Rubin, and E. De Ritter.

The presentation of the material is novel. The book is not divided into chapters. The table of contents gives the general headings and the subdivisions for each subject. The first page of the section devoted to one subject gives a more detailed table of contents. A bibliography is given at the end of each section. Complete author and subject indexes are given at the end of the book.

The description of and the application of statistical procedures are comprehensive. Of the 712 pages of text material, 166 pages are devoted to the section on statistical methods in vitamin research.

Although the editor states that the chapter on statistical methods is written so that even the inexperienced may make use of modern statistical tools, one gets a firm conviction that the average experimenter would be wise to enlist the collaboration of a statistical specialist on any major problem. The chapter includes a glossary of statistical symbols that covers eight pages.

The contribution on the clinical science of malnutrition and the collective presentation of clinical-chemical methods useful in the diagnosis of deficiency conditions should be especially valuable to physicians.

Microbiological methods for the estimation of vitamin B_{12} are included, and the use of chromatographic and ion exchange procedures for the determination of carotene and of panthenol and pantothenate, respectively, are described. The descriptions of methods are thorough and clear, and the additional notes and observations are helpful. This book should be available to all who are interested in the vitamins.

BOOKS RECEIVED

Organic Syntheses. Vol. 31. R. S. SCHREIBER, Editor-in-Chief. John Wiley and Sons, Inc., New York, 1951. vi + 122 pp. 15 x 23.5 cm. Price $2.75.

Aids to Pharmaceutical Calculations. By MARY E. BOLTON. Bailliére, Tindall and Cox, London, 1951. vii + 96 pp. 10.5 x 16.5 cm. Price $2.

American Pharmacy. 3rd ed. RUFUS A. LYMAN, Editor-in-Chief. J. B. Lippincott Company, Philadelphia, 1951. xiii + 505 pp. Illustrated. 19 x 26 cm. Price $8.

Textbook of Pharmacognosy. 2nd ed. By T. E. WALLIS. J. & A. Churchill Ltd., London, 1951. xi + 556 pp. Illustrated. 15 x 23.5 cm. Price 35*s*.

Hydrogenation of Fatty Oils. By H. I. WATERMAN. Elsevier Publishing Company, Houston, Tex., 1951. ix + 254 pp. 15.5 x 23.5 cm. Price $6.50.

Biological Antagonism. By GUSTAV J. MARTIN. The Blakiston Company, Philadelphia, 1951. vii + 516 pp. 16 x 23.5 cm. Price $8.50.

Organic Chemistry. By J. P. WIBAUT. Elsevier Publishing Company, Houston, Tex., 1951. xiv + 660 pp. 18 x 26 cm. Price $9.

Calendar of the Pharmaceutical Society of Great Britain, 1951–1952. The Pharmaceutical Society of Great Britain, London, 1951. 308 pp. 14.5 x 22.5 cm. Price 12*s*. 6*d*.

Pharmacopoea Danica 1948, Addendum 1951. Arnold Busck International Booksellers, Copenhagen, 1951. 142 pp. 16.5 x 24 cm.

Outline of Fundamental Pharmacology. By DAVID FIELDING MARSH. Charles C Thomas, Publisher, Springfield, Ill., 1951. xxxi + 219 pp. 16 x 23.5 cm. Price $6.

Physiology and Pharmacology for Pharmaceutical Students. 3rd ed. By HAROLD HAYDEN BARBER. Williams & Wilkins Co., Baltimore, 1951. x + 622 pp. Illustrated. 15 x 22.3 cm. Price $6.50.

A Dictionary of Antibiosis. Compiled by LEONARD KAREL and ELIZABETH SPENCER ROACH. Columbia University Press, New York, 1951. 373 pp. 16 x 24.3 cm. Price $8.50.

Phase Microscopy—Principles and Applications. By ALVA H. BENNETT, HELEN JUPNIK, HAROLD OSTERBERG, and OSCAR W. RICHARDS. John Wiley and Sons, Inc., New York, 1951. xiii + 320 pp. Illustrated. 15.5 x 23.5 cm. Price $7.50.

Shortly before Christmas in 1867, Gustavus, Edward and Otto Mallinckrodt, after long and careful planning, established a chemical works on the family farm in St. Louis. Its start was not overly auspicious, in the face of such exciting local events as the building of the *great Eads Bridge*, the launching, by George Francis Train and Elizabeth Cady Stanton, of the *women's suffrage movement* in St. Louis; the organization of the *mounted police force;* the burning of the lavish *Lindell Hotel*, the thrilling appearance of *Edward Payson Weston*, noted pedestrian, who gave an exhibition of his talents in the old Mercantile Library Hall after completing a walking tour of 1,316 miles from Portland, Maine, to Chicago; the performances of *Lotti's German Opera;* and the excitement of the country over the report that soldiers, sent to take possession of *Alaska*, were in destitute condition.

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